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Forced Degradation Study of Racecadotril: Effect of Co-solvent, Characterization of Degradation Products by UHPLC-Q-TOF-MS/MS, NMR and Cytotoxicity Assay

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Abstract. Racecadotril, an enkephalinase inhibitor, was subjected to hydrolysis (acidic and alkaline), oxidation, photolysis and thermal stress, as per ICH specified conditions. The drug showed extensive degradation under acidic, basic hydrolysis and oxidative stress conditions whereas, it was stable under other stress conditions. A total of seven degradation products (DPs) were observed. The chromatographic separation was optimized on Acquity HSS Cyano (100 × 2.1 mm, 1.8 μ) column using 0.1% formic acid and acetonitrile as mobile phase in gradient mode. Six DPs were characterised by LC–MS/MS and DP1 by GC–MS. The major DPs (DP 2 and DP 5) were isolated and characterised by NMR. This is a typical case of degradation where co solvent methanol reacts with racecadotril leading to the formation of pseudo DPs, DP 6 and DP 5. Interestingly the MS/MS spectra of protonated drug, DP 4 and DP 7 showed product ions which were formed due to intramolecular benzyl migrations. In vitro cytotoxic activity studies on isolated DP 2 and DP 5 revealed that the former has no cytotoxic nature, whereas the latter has potential pulmonary and hepatic toxicity.

Keywords: Forced degradation; LC–MS/MS; Benzyl-benzyl interactions; GC–MS; Cytotoxic assay.

1. Introduction

Racecadotril (RACE) [(RS)-benzyl N-[3-(acetylthio)-2-benzylpropanoyl] glycinate] is an antidiarrheal drug which acts by inhibiting enkephalinase peripherally and suitable for use in infants and young children. It is rapidly converted in the body to thiorphan, a potent enkephalinase inhibitor. Enkephalins are endogenous opioid peptides secreted by myenteric and sub mucosal neurons in the digestive tract. The mechanism of action of enkephalins involves activation of the δ opioid receptor which inhibits the secretion of chlorine ions and fluids leading to the loss of fluids and electrolytes during diarrhea [1], [2] and [3]. Stress stability is an integral part of the drug development process and explains several factors that affect the expiration dating of drug products, including the chemical and physical stability during the preclinical formulation stages, process development, packaging development, and post marketing life. Benefits of structural characterization of degradation products includes, understanding of their origin resulting into ways for their control during drug synthesis or formulation development. In addition, the degradation products can either be isolated or be synthesized and subsequently their toxicity can be evaluated with the help of cytotoxicity tests like MTT assay which helps in drug discovery and development research [4].

A few analytical methods have been reported on RACE, which includes stability indicating assay methods [5], [6], [7] and [8], bioanalytical methods [9] and [10]. The hydrolytic degradation behavior of RACE was studied under acidic and alkaline conditions by Pawan et al. [11]. However, no systematic study on the degradation behavior of racecadotril and characterization of all the DPs

formed has been reported. In general stress studies are limited to non-volatile DPs, whereas the identification and characterization of volatile DPs is not done in routine. The study of volatile DPs along with non-volatile DPs also helps in establishment of mass balance. Hence, the purpose of the present study is to develop a stability indicating assay method for RACE and to characterize the degradation products (volatile and non-volatile) formed as per the International Conference on Harmonization (ICH) recommended stress conditions using LC-ESI–MS/MS, GC–MS, NMR and cytotoxicity evaluation of isolated major degradation products using MTT assay. The results of the present study may also be helpful for the assessment of the quality of stored products that have expired or on the edge of getting expiration.

2. Experimental

2.1. Drug and reagents

Pure racecadotril (99.56% purity) was obtained as a gift sample from Symed Labs Hyderabad, India. UPLC grade methanol, acetonitrile and hydrogen peroxide were purchased from Merck (Mumbai, India). All analytical grade reagents: ammonium formate, formic acid, sodium hydroxide, hydrochloric acid, and 30% (w/w) hydrogen peroxide were purchased from SD Fine Chemicals Pvt. Ltd., (Mumbai, India). LC grade water was prepared by filtrating through a Millipore Milli-Q- plus system (Merck Millipore, Billerica, Massachusetts, United States). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Sigma–Aldrich. Neuro-2a (mouse neuroblastoma cells), A549 (adenocarcinomic human alveolar basal epithelial cells) and Hep G2 (human liver carcinoma cell) cells were obtained from American Type Culture Collection (Manassas, VA, United States).

2.2. Instrumentation

For LC–MS analysis, an Agilent Infinity 1290 series instrument (Agilent Technologies, Santa Clara, California, USA) coupled to quadrupole time-of-flight (Q-TOF LC–MS 6540) equipped with an electrospray ionisation (ESI) source was used. The data acquisition was under the control of Mass Hunter workstation software. The ESI source conditions were optimized as follows: fragmentor voltage, 150 V; capillary voltage, 4000 V; skimmer, 65 V; nitrogen was used as drying (350 °C; 10 L/min) and nebulising (40 psi) gas. For full scan MS mode, the mass range was set at m/z 50–2000. Ultra-high purity nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions and at an average of 20–25 scans.

Identification of volatile degradation product was carried out on an Agilent 6890 gas chromatograph (Agilent Technologies Inc., Santa Clara, California, USA) equipped Mass Selective Detector (MSD, 5973), autosampler (G2614), and electron impact (EI) ionisation source controlled by ChemStation software.

1D and 2D NMR experiments were performed on a 500 MHz NMR (AVANCE III HD-500, Bruker, Billerica, Massachusetts, United States) spectrometer using CDCl3 as solvent. 1H chemical shift values were reported on the δ scale in ppm relative to TMS (δ = 0.00 ppm) as internal standard. The data acquisition and processing of NMR spectra was done using Top spin software (3.2 version).

Photolytic studies were carried out in a photostability chamber (Osworld OPSH-G-16-GMP series, Osworld Scientific Equipments Pvt. Ltd. India) set at 40.0 ± 5.0 °C/75.0% relative humidity (RH) ±3.0% RH and equipped with an illumination bank on inside top, consisting of a combination of two black light ultraviolet lamps and four white fluorescent lamps in accordance with option 2 of the ICH guideline Q1B. The thermal degradation studies were carried out in the Osworld laboratory oven (Osworld scientific Pvt. Ltd. India). All pH measurements were carried out on a pH meter (Metrohm Schweiz AG, 780 pH meter, Germany) with Epson printer Lx-300 t and weighing was done on a Sartorius balance (CD 225 D, 22308105 Germany).

2.3. Forced degradation studies

Forced degradation of RACE was carried out on the bulk drug as per ICH guidelines [12] and [13]. RACE was subjected to stress hydrolytic degradation study by refluxing 30 min in acidic (1.0N HCl), and 5 min in alkaline (0.01N NaOH) conditions at room temperature using acetonitrile and methanol

as co solvents. The optimized oxidative, photolytic, and thermal stress conditions are given in Table 1. The drug solutions were prepared at 1.0 mg/mL concentration for all stress samples.

2.4. Sample preparation

All the stressed samples (hydrolytic, oxidative, thermal, and photolytic stress) were neutralized and diluted with mobile phase and filtered through 0.22 μ membrane filter before LC–MS analysis.

2.5. Cytotoxicity assay

Neuro-2a, A549 and Hep G2 cells were seeded at a density of 5 × 103 cells per well in 96-well plate. After 24 h incubation in CO2 incubator, DP 2 and DP 5 were added in different concentrations over a range of 3.125–100 μM, incubated further for 48 h. 100 μ L of MTT at 0.5 mg/mL concentration was added to the above sample and incubated for 4 h. Then the media with MTT was removed and the resulted formazan crystals were dissolved by addition of 100 μL DMSO and absorbance was measured at 570 nm using multi detection plate reader (Spectramax M4, Molecular devices, USA).

2.6. Isolation of major degradation products by column chromatography

For isolation of major degradation products (DP 1, DP 2 and DP 5) initially base hydrolytic condition was used, but the degradation products formed were not stable and within less time they are converted into DP 1. Therefore acidic hydrolytic condition was tried by dissolving 300 mg of RACE in 5 mL of methanol, and added 15 mL of 1N HCl. Final volume was made up to 25 mL with methanol and refluxed for 4 h at 80 °C, where DP 1, DP 2 and DP 5 were formed in major quantity. The DP 1 was volatile liquid and it got evaporated along with solvents (methanol and 1N HCl) and was received in collecting flask leaving remaining degradation products in rotating flask, which were solidified and loaded on to the silica column. Fractions containing >99% of DP 5 (eluted with 40% ethyl acetate in hexane), DP 2 (eluted with 70% ethyl acetate in hexane) were pooled together; concentrated on rotavapor to remove ethyl acetate and hexane using a rotary evaporator. DP 2 and DP 5 were obtained as white powder with chromatographic purity of 99.10% and 99.60% respectively.

3. Results and discussion

3.1. Chromatographic separation

The preliminary experiments were done to develop a chromatographic method capable of resolving the drug and its stress degradation products. Initial trials were performed on Acquity HSS SB C18 (100 × 2.1 mm, 1.8 μ) using 0.1% formic acid as aqueous phase and acetonitrile, methanol as organic phase in gradient elution mode. Better separation and peak shape were observed with formic acid and acetonitrile, but resolution for the critical pair, DP 4 and RACE was less (1.3). In order to improve the resolution of critical pair, instead of changing gradient method and mobile phase components, different columns i.e. Acquity HSS T3 (100 × 2.1 mm, 1.8 μ), Acquity CSH C18 (100 × 2.1 mm, 1.7 μ) and Acquity HSS Cyano (100× 2.1 mm, 1.8 μ) were tried and better separation was found with HSS Cyano column (resolution is 2). A linear gradient program was set as follows: 0/30, 2/30, 5/70, 7/70, 8/30, and 10/30 at flow rate of 0.3 mL/min. Chromatogram was monitored at 215 nm, column temperature was 30 °C and injection volume was 2 μL. With the developed method RACE and its degradants were well separated with adequate resolution and symmetric peak shape.

For characterisation of volatile DP, Zebron ZB-624 (Phenomenex) column (30 m × 0.25 mm × 1.40 μ) was used with an initial GC oven temperature of 35 °C (2 min hold). The temperature was raised at the rate of 8 °C/min to 220 °C, where it was maintained for 5 min; injector temperature was set at 220 °C and the MSD interface was set at 230 °C. The standard split/splitless liner was used in split injection mode (1:50). Hydrogen was used as carrier gas at a constant flow rate of 1.2 mL/min.

3.2. Degradation behavior of RACE

The degradation behavior of RACE was studied using LC–MS under various forced degradation conditions. Sufficient degradation was observed in all conditions except in photolysis and thermal degradation where drug was found to be stable. When methanol was used as co solvent in hydrolytic degradation (acidic and basic) pseudo DPs, DP 5 and DP 6 were formed. This may be attributed to participation of methanol in the degradation chemistry by acting as a nucleophile to react with

electrophilic sites or intermediates in the degradation pathways (Scheme S4a,b, Supporting information). The overlay of chromatograms of all stress degradation samples are given in Fig. 1.

3.3. Characterization of RACE and its DPs by LC–MS/MS

RACE and all the DPs (DP 1–7) were well separated by LC and they exhibited abundant protonated molecular ions ($[M+H]^+$) in positive ionization mode. MS/MS spectra of the $[M+H]^+$ ions of RACE and DPs were recorded to obtain structural information. The fragmentation patterns were obtained based on MS/MS experiments and accurate mass measurements from HRMS data. A total of seven DPs were formed but only six DPs were characterized using LC-ESI–MS/MS experiments as DP 1 was not detected under ESI, APCI and APPI ionisation conditions and the proposed structures of DPs and their elemental compositions are given in Scheme 1 and Table S1, Supporting information. However, DP-1 was analyzed by GC-EIMS which will be discussed later.

The ESI–MS/MS spectra of protonated RACE and those of DPs are shown in Fig. 2, and the proposed fragmentation patterns of their $[M+H]^+$ ions are shown in Schemes S1–S4, Supporting information. The elemental composition of all the ions of RACE and the DPs are summarized in Table S1, Supporting information.

3.3.1. MS/MS of $[M+H]^+$ of RACE (m/z 386)

To study the degradation behavior of RACE, the ESI–MS/MS spectrum of its protonated molecule (m/z 386) was examined. The spectrum showed product ions at m/z 344 (loss of ketene, CH_2CO from m/z 386), m/z 326 (loss of water molecule from m/z 344), m/z 278 (loss of C_6H_7O from m/z 386), m/z 269 (by migration of benzyl group to another benzyl group [14] followed by loss of $C_2H_5NO_2$ from m/z 344), m/z 236 (loss of C_7H_8O from m/z 344), m/z 235 (loss of H_2S from m/z 269) m/z 207 (loss of CO from m/z 235), m/z 179 (loss of C_2H_3NO from m/z 236), m/z 145 (loss of H_2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C_3H_2O from m/z 145), m/z 65 (loss of C_2H_2 from m/z 91) and m/z 76 (2-aminoacetic acid moiety). The elemental compositions of all these fragment ions (Scheme S1, Supporting information) have been confirmed by accurate mass measurements and are given in Table S1, Supporting information.

3.3.2. Characterization of DPs

Initially, LC-ESI–MS/MS analysis was tried in both positive and negative modes. All the degradants showed intense $[M+H]^+$ peaks in positive ion mode and very low intensity peaks in negative mode. Thus, the analysis was carried out in positive ionization mode and the MS/MS spectra of the degradation products are shown in Fig. 2. Most plausible structures have been proposed for all the DPs based on the m/z values of their $[M+H]^+$ ions and the MS/MS data in combination with elemental compositions derived from accurate mass measurements. Comprehensive characterization of all the DPs is discussed below.

The LC-ESI–MS/MS spectrum of $[M+H]^+$ of DP 2 (m/z 254, $C_{12}H_{16}NO_3S^+$, Rt 2.15 min) shows the product ions at m/z 236 (loss of water), m/z 179 (loss of C_2H_3NO from m/z 236), m/z 145 (loss ofH_2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C_3H_2O from m/z 145), m/z 65 (loss of C_2H_2 from m/z 91) and m/z 76. All these fragment ions are structure indicative and highly compatible with the structure, 2-(2-benzyl-3-mercaptopropanamido) aceticacid (Scheme S2, Supporting information). The formation of DP2 under base and acid hydrolytic conditions can be explained by hydrolysis of ester and thio ester bonds to form carboxylic acid and thiol groups (Scheme S4a,b, Supporting information).

The degradation product DP 3 at m/z 296 $[M+H]^+$; $C_{14}H_{18}NO_4S^+$ was eluted at 2.46 min. It can be formed by hydrolysis of ester group to form carboxylic acid (Scheme S4a,b, Supporting information). Its MS/MS spectrum shows product ions at m/z 254 (loss of ketene, CH_2CO from m/z 296), m/z 236 (loss of water molecule from m/z 254), m/z 208 (loss of CO from m/z 236), m/z 179 (loss of C_2H_3NO from m/z 236), m/z 145 (loss of H_2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C_3H_2O from m/z 145) and an intense base peak at m/z 76. The formation of an intense peak at m/z 76 indicates that 2-aminoacetic acid moiety of the drug is intact in the DP. The observed fragmentation of protonated DP 3, supported by accurate mass measurements (Table S1, Supporting information), is found to be highly consistent with the proposed structure 2-(3-(acetylthio)-2-benzylpropanamido) acetic acid (Scheme S2, Supporting information).

The DP 4 at m/z 344 ($[M+H]^+$, $C_{19}H_{22}NO_3S^+$), formed by hydrolysis of thio ester group (Scheme S4a,b Supporting information), was eluted at 6.20 min. Its MS/MS spectrum shows product ions at m/z 269 (by migration of benzyl group on to another benzyl group [14] followed by loss of $C_2H_5NO_2$ from m/z 344), m/z 236 (loss of C_7H_8O from m/z 344), m/z 235 (loss of H_2S from m/z 269), m/z 207 (loss of CO from m/z 235), m/z 179 (loss of C_2H_3NO from m/z 236), m/z 145 (loss of H_2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C_3H_2O from m/z 145), m/z 65 (loss of C_2H_2 from m/z 91) and m/z 76 (2-aminoacetic acid moiety). Based on these data combined with accurate mass measurements (Table S1, Supporting information), DP 4 was identified as benzyl 2-(2-benzyl-3-mercaptopropanamido) acetate (Scheme S1, Supporting information).

The ESI–MS/MS spectrum (Fig. 2) of protonated DP 5 (Rt 3.10, m/z 268) shows the product ions at m/z 250 (loss of H_2O), m/z 236 (loss of methanol from m/z 268), m/z 208 (loss of CO from m/z 236), m/z 179 (loss of C_2H_3NO from m/z 236), m/z 145 (loss of H_2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C_3H_2O from m/z 145), and m/z 65 (loss of C_2H_2 from m/z 91). The formation of an intense ion at m/z 90 indicates the presence of 2-aminoacetic acid methyl ester moiety in the degradation product. From the fragmentation studies (Scheme S2, Supporting information), DP 5 was identified as methyl 2-(2-benzyl-3-mercaptopropanamido) acetate. The elemental compositions of $[M+H]^+$ of DP 5 and its fragment ions have been confirmed by accurate mass measurements and are given in Table S1, Supporting information.

The MS/MS spectrum of $[M+H]^+$ of DP 6 (Rt 3.65 min, m/z 310, $C_{15}H_{20}NO_4S^+$) is given in Fig. 2. It shows product ions at m/z 278 (loss of methanol moiety from m/z 310, indicating that (2-(3-(acetylthio)-2-benzylpropanamido) ethylidyne) oxonium moiety is intact). The ion at m/z 268 (loss of CH_2CO from m/z 310), indicates the presence of methyl 2-(2-benzyl-3-mercaptopropanamido) acetate moiety, m/z 236 (loss of methanol from m/z 268), m/z 221 (loss of C_2H_3NO from m/z 278), 208 (loss of CO from m/z 236), m/z 179 (loss of C_2H_3NO from m/z 236), m/z 145 (loss of H_2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C_3H_2O from m/z 145). Based on MS/MS experiments and accurate mass measurements (Table S1, Supporting information), the structure of DP 6 can be assigned as methyl 2-(3-(acetylthio)-2-benzylpropanamido) acetate (Scheme S2, Supporting information).

The ESI–MS spectrum of DP 7 (Rt 4.40 min) shows the $[M+H]^+$ ion peak at m/z 402 with an elemental formula, $C_{21}H_{24}NO_5S$. A mass difference of 16 Da indicates that degradant was formed by an addition of one oxygen atom. MS/MS spectrum of protonated DP 7 displayed product ions at m/z 384 (loss of water molecule), m/z 360 (loss of ketene), m/z 344 (loss of water from m/z 360), and m/z 296 (loss of C_7H_6O from m/z 402). The ion at m/z 296 can be formed through a McLafferty rearrangement involving the hydroxyl hydrogen at the benzylic carbon and the carbonyl group as shown in the Scheme S3, Supporting information. This also supports the hydroxylation of benzylic carbon under oxidative conditions to form DP 7. It also shows the fragment ions at m/z 285 (formed from m/z 360 by migration of benzyl group on to another benzyl group [14] followed by loss of $C_2H_5NO_2$), m/z 251(loss of H_2S from m/z 285), m/z 149 (fromm/z 360), m/z 107 (loss of CH_2CO from m/z 149), m/z 91 (from m/z 342), m/z 76 (loss of $C_{19}H_{18}O_3S$ from m/z 402) and m/z 65 (loss of C_2H_2 from m/z 91). From MS/MS data and accurate mass measurements (Table S1, Supporting information), the structure of DP 7 can be assigned as benzyl 2-(2-(acetylthiomethyl)-3-hydroxy-3-phenylpropanamido) acetate.

The isolated DP 1 (Rt 13.65 min, S9 Supporting information) was analyzed by GC-EIMS. The EI spectrum of the sample matched with that of benzyl alcohol from NIST library (Fig. 5). This DP can be easily formed from the drug under hydrolytic conditions as shown in Scheme S4 a,b, Supporting information.

3.4. NMR structural characterization of major degradation products (DP 2 and DP 5)

^1H NMR chemical shifts (δ), and ^{13}C NMR chemical shift values are presented for DP 5 and RACE in Table 2. NMR studies of DP 5 reveals that it has 17 hydrogens and 13 carbons. Absence of singlet at δ 2.32 ppm (3H), and presence of triplet at δ 1.66 ppm (SH) confirms the hydrolysis of thioester group. This is also evidenced by absence of a peak at δ 195.93 (Cdouble bond; length as m-dashO) in ^{13}C NMR spectrum of DP 5. ^1H and ^{13}C NMR spectra of DP 5 also show the absence

of proton and carbon peaks related to benzyl group and presence of a methyl group at δ 3.73 ppm. DEPT135 and 13C NMR experiments verified that DP 5 contains 1-CH3 (primary), 3-CH2 (secondary), 6-CH (tertiary) and 3-C (quaternary) carbons. DEPT135 spectrum of DP 5 shows presence of new peak at δ 52.28 (18CH3) and absence of peaks related to 20, 21, 23, 24, 25, 26 and 27 carbon atoms which were earlier seen in DEPT135 spectra of RACE. The single bond carbon proton heteronuclear correlation experiments (HSQC) are shown in Fig. 4. HSQC spectrum of DP 5 shows the absence of correlations of CH3 (20), CH2 (21) and CH (23, 24, 25, 26, 27) which were observed in RACE HSQC spectrum. The observations from 1H, 13C, DEPT135 and HSQC NMR spectra confirm that DP 5 is methyl 2-(2-benzyl-3-mercaptopropanamido) acetate (Fig. 3).

The structure of DP 2 was also confirmed by 1H NMR experiments. 1H NMR (SI) values in CDCl3, δ (ppm) 10.32 (s, 1H) 7.39–7.257 (m, 6H, 5 are of aromatic protons and one is CDCl3 proton), 6.06 (s, 1H), 4.20–4.15 (dd, 1H), 3.99–3.95 (dd, 1H), 3.08–3.04 (m, 1H), 2.97–2.94 (m, 1H), 2.71–2.69 (m, 1H), 2.67–2.65 (m, 1H) and 1.771.74 (t, 1H). The 1H NMR spectrum of DP 2 and DP 5 were compared for characterization. The most characteristic peak at δ 10.32 (s, 1H) of carboxylic acid and loss of a peak at δ 3.73 (s, 3H) of methyl group confirms the structure of DP 2 as 2-(2-benzyl-3-mercaptopropanamido) acetic acid.

3.5. Cytotoxicity assay

The toxicity of degradation product, DP 1(benzyl alcohol) was extensively studied and reported in the literature [15], [16], [17], [18], [19], [20], [21] and [22]. Hence, cytotoxicity tests for the isolated degradation products DP 2 and DP 5 were evaluated in the present study. From absorbance values, percentage inhibition was calculated from equation (% Inhibition = (1 − ASample/AControl) × 100), and IC50 values were calculated from concentration vs percentage inhibition graph (GraphPad Prism software). The results indicated (Fig. 6) that the compound DP 5 has significant cytotoxicity (on A549 and Hep G2 cells), whereas DP 2 is nontoxic. The IC50 values of DP 5 on A549 and Hep G2 cells were found to be 58.42 μM (±7.15), 82.6 μM (±9.01), respectively.

4. Conclusion

Forced degradation studies on racecadotril were performed as per ICH guidelines. A total of seven DPs were identified and characterized using LC–MS/MS and GC–MS. This is a typical case of degradation where co solvent methanol reacts with RACE leading to the formation of pseudo DPs, DP 6 and DP 5. Major DPs (DP 2 and DP 5) were isolated and their structures were confirmed by MS/MS and NMR. The cytotoxic activity study of these two DPs revealed that DP2 to be non-toxic whereas DP 5 has potential pulmonary and hepatic toxicity.

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The Impact of Ethics on the Design and Conduct of Acupuncture Clinical Trials

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Abstract. Racecadotril, an enkephalinase inhibitor, was subjected to hydrolysis (acidic and alkaline), oxidation, photolysis and thermal stress, as per ICH specified conditions. The drug showed extensive degradation under acidic, basic hydrolysis and oxidative stress conditions whereas, it was stable under other stress conditions. A total of seven degradation products (DPs) were observed. The chromatographic separation was optimized on Acquity HSS Cyano (100 × 2.1 mm, 1.8 μ) column using 0.1% formic acid and acetonitrile as mobile phase in gradient mode. Six DPs were characterised by LC–MS/MS and DP1 by GC–MS. The major DPs (DP 2 and DP 5) were isolated and characterised by NMR. This is a typical case of degradation where co solvent methanol reacts with racecadotril leading to the formation of pseudo DPs, DP 6 and DP 5. Interestingly the MS/MS spectra of protonated drug, DP 4 and DP 7 showed product ions which were formed due to intramolecular benzyl migrations. In vitro cytotoxic activity studies on isolated DP 2 and DP 5 revealed that the former has no cytotoxic nature, whereas the latter has potential pulmonary and hepatic toxicity.

Keywords: Forced degradation; LC–MS/MS; Benzyl-benzyl interactions; GC–MS; Cytotoxic assay.

1. Introduction

The consideration of bioethics is an integral process in the design and conduct of a clinical trial. Currently, peer reviewed journals require a statement that the submitted research has been assessed and approved by an institutional review board. Researchers often overlook ethical issues until they are required to submit such an application for approval. While there may have been an implicit understanding and acknowledgment of ethical issues, the application process can help clarify issues and may lead to consideration of different design options. Furthermore, ethical issues often arise during the conduct of research and therefore a discussion on the contribution of ethics to acupuncture research seems additionally warranted. This paper will outline some general issues associated with ethical research and how they may impinge on the design and implementation of acupuncture research.

2. Ethics and health research

Western medical ethics arose from the clinical practice of medicine and had its ethical origins in the Hippocratic code that sought to regulate the practice of its practitioners. During the development of medicine the focus of ethics shifted to medical etiquette, with the development of customs relating to dealing with patients and other practitioners. Practitioners were often loath to reveal their treatment and there was reluctance amongst them to criticise one another.1

Following the Nuremberg trials of 1946, the ethical principles for human experimentation were defined in what was known as the Nuremberg code. It arose from the abuse of medical research in the

Nazi concentration camps. This was later incorporated into the World Medical Association (WMA) Declaration of Helsinki that was to form the basis for the development of guidelines for human experimentation.2 Since its adoption in 1964 by the WMA, it has undergone a number of revisions, the most recent being in 2000.3 As clinical research accelerated, many institutions in the 1960s and 70s looked to the establishment of institutional review boards. They used the Declaration of Helsinki as a basis for evaluating ethical issues such as informed consent, protection from harm, weighting of hazards and benefits, coercion to participate, and the protection of the subject's privacy.

Chinese Medicine also had its ethical codes of practice whereby illness was seen as arising from a departure from laws of nature and society. Confucianism especially, with its emphasis of human relations and obligations towards fellow human beings, had a definite influence on the development of ancient medical ethics.4 With the spread of Chinese medicine to the West, the development of ethical concerns has continued, especially in relation to professional practice ethics in a Western society.5

As a profession, acupuncturists are being called to validate their claim that acupuncture has therapeutic value. They are ethically bound as a group, to respond to the request by designing and participating in methodologically sound clinical research. It would be unethical to dismiss the request and continue to offer treatment based on unsubstantiated claims that have not been externally validated.6 In addition, researchers are ethically obliged to improve upon and develop new methods of treatment. The Declaration of Helsinki3 clearly identifies the need to investigate scientifically unproven practices such as acupuncture, that nevertheless have a long history of clinical success.

> In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgement it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published.

3. Institutional review boards

Institutional Review Boards (IRB) or Human Research Ethics Committees as they are termed in the United States, were established to ensure that all research undertaken by staff and students of an institution conform to the highest ethical standards. The three fundamental principles underlying the ethical administration of research are respect of persons, respect for justice and beneficence.7 The role of the IRB extends to protect not only the interests of the participants of the research, but also the researchers and the institution. With the introduction of acupuncture into higher education and the increasing attraction for acupuncture practitioners to undertake research, the need to understand the ethical concerns of research and the process of gaining ethical approval is imperative. Furthermore, novice researchers can gain an understanding of the issues that confront the IRB when an acupuncture research proposal is submitted, while the IRB can gain an understanding of the peculiar ethics that may be associated with acupuncture research methodology when an application is evaluated. This last point is especially relevant when many IRBs may not have had experience with evaluating acupuncture proposals and may turn down a good proposal because they fail to understand the methodology, or the terminology, in the application. It is incumbent on the acupuncture researchers, whether they are novices or experienced researchers, to make sure their application has every opportunity to be approved. IRB members come from a wide variety of backgrounds and often include members of the public. It is therefore necessary to communicate in plain simple language free from jargon. Technical terminology should be kept to a minimum and a glossary provided if necessary. On the other hand, the IRB will also include experienced researchers who may be unfamiliar with or biased against acupuncture, and it is important that the application communicates in terms they are familiar with. Chinese medicine terms should be limited and clearly defined or alternative expressions found. For example the acupuncture term deqi could be replaced by the term needling sensation.

Another possible barrier to acupuncture research, especially for independent acupuncture colleges, could be the lack of access to a IRB. The Declaration of Helsinki3 clearly directs that all research should be channelled through a IRB for ethical consideration.

> This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be in conformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.

The submission of a research application to a IRB is obviously a problem for small acupuncture colleges who are not affiliated with orthodox institutions and do not have the resources or infrastructure to establish a IRB. The most obvious solution would be to collaborate with established researchers who are linked to an institution, such as a hospital or University, that do have an established IRB. Another option would be to approach a IRB independently. The IRB may be willing to assess a proposal and monitor the research for a fee. This may lead to future research collaboration with future research. A third, less attractive option, would be to establish an IRB. At the University of Technology, Sydney (UTS) where the author resides, a small IRB was formed to assess independent undergraduate research projects. The larger institutional (UTS) IRB was kept informed of the process and any dubious ethical issues brought to their attention. When pursuing this option, the IRB administrator at the local larger institution, may well be willing to help train the IRB being established at the acupuncture college. The advantage is that an acupuncture college based IRB is likely to become more knowledgeable in basic concepts of Chinese medicine in a shorter period of time, as well as being more sensitive to design issues that are particularly relevant to acupuncture research.

4. Developing an ethical research agenda

It is also important to consider how research will impact on community health and the contribution that the research will make to the discipline. Research needs to have outcomes that have significance for both the field of research and the wider community. The identification of diseases or illnesses that are in need of better therapies, and which acupuncture has been seen as a viable but untested treatment option, is a necessary first step in establishing a research agenda.8 Edwards9 has suggested that the prospective researcher needs to reflect on such issues. Why are we doing the research? Are we using systematic investigations designed to develop and contribute to the knowledge in the field? What will it produce for the investment? What will it contribute to the consumers of the research? Reflective questions such as these focus on the ethical heart of research. Researchers who are unable to give a satisfactory answer to such questions need to re-evaluate their goals and consider the health of the community, as well as their own interests.

IRBs also require that the research methodology is the most appropriate for the project and that it addresses the research question in a scientific manner. The adoption of a systematic research protocol, such as the US Food and Drug Administration (FDA) approach to clinical trials, allows the research team to develop the most appropriate and rigorous methodology for the research area.10 Given the high financial and human costs associated with implementing a clinical trial, it is imperative that the limited research resources are utilised in an ethical manner.[11] and [12] To proceed to a large-scale phase III clinical trial without some evidence or understanding of the research issues should be considered unethical, as well as a potentially wasteful venture.

5 . Ethical constraints associated with methodology

Constraints regarding acupuncture methodology, such as the difficulty of double blinding and the choice of a control, need to be identified and communicated to the IRB. The difficulty of double

blinding in an acupuncture trial has been well recognised yet IRB members may have little understanding of this issue which may lead to rejection of the application.13 Strategies to overcome the problems associated with single blinding may include minimizing bias by limiting or standardizing interaction, and blinding assessors. The strategies need to be made explicit when writing the application.10

The issue of whether or not to use a placebo control treatment and under what circumstances remains a vexing ethical issue.[14], [15] and [16] Rothman17 in reviewing the ethical concerns associated with the use of a placebo argues that

> 'every patient-including those of a control group, if any- should be assured of the best proven diagnostic and therapeutic method.' This statement effectively proscribes the use of a placebo as control when a 'proven' therapeutic method exists. The declaration [of Helsinki] also directs that a study that violates its precepts should not be accepted for publication… there is no straightforward way to estimate how many trials are undertaken that involve the unethical use of placebos.

This raises some interesting questions concerning the use of a placebo in acupuncture research. For example, it seems reasonable and ethical to demonstrate that the efficacy of acupuncture is not entirely due to the placebo effect. Conversely, it may not be ethical to treat a group of ill or diseased subjects with a placebo, despite them having given informed consent.

Hammerschlag15 in reviewing the ethical concerns associated with acupuncture control treatments classified the control options into five categories. The no treatment control (either wait list or no treatment) involves either delaying or denying of acupuncture treatment. The ethical issue associated with this option is the concern of whether the delay or denial of treatment would have a critical impact on the disease process. If the disease is greatly affected by the delay or denial of treatment then this method of control could be seen as ethically dubios. If however, the delay or denial of treatment had minimal effect on the disease, in other words the disease was of a stable and chronic nature, the ethical concerns would be minimal. A possible strategy to circumvent the issue would be to offer treatment, acupuncture if the outcome was positive, or standard medical care if the outcome was negative, after completion of data collection.

The second category, the acupuncture versus biomedical care model, was seen as being ethical in that there is an 'intent to treat' all subjects in the trial. This means that in both treatment arms, the acupuncture and the standard care, there was intent to treat the subject. However as acupuncture could be seen as an experimental procedure, the intent to treat does not necessarily equate to receiving appropriate treatment. Again, as in the no treatment category, either standard care or a course of acupuncture could be offered to the subject on completion of the trial. The issue of the delay or denial of treatment in the acupuncture group is also present in this model.

The third category was the acupuncture plus standard care versus standard care only model. This is the most ethical of the five control options in that there is no attempt to deny subjects effective, or partially effective, standard treatment. Fundamental to this approach is the recognition that the standard treatment has been previously validated as an effective or partially effective treatment. The 'intent to treat' is common to both groups.

The next two categories, acupuncture versus placebo (non-invasive treatment) and acupuncture versus sham needling (invasive treatment) are different from the previous three categories in that there is an intent to deceive subjects. The justification for the deception in the form of a placebo treatment is to control for 'expectations of benefit'. Again the issue of delay or denial of treatment is present.

In reviewing these five control options, two ethical issues become apparent. The first relates to the concept that acupuncture is still perceived as an experimental therapy and the second to the delay or denial of standard medical care. If we take the perspective that acupuncture is an experimental treatment then ethically we are required to ensure standard treatment, albeit an imperfect treatment, is not denied to the subject. This could be given at the completion of the trial (for the no treatment, the acupuncture versus biomedical care and the placebo and sham needling groups) or concurrently (for the acupuncture versus biomedical care group). Furthermore, it could be argued on ethical grounds

that the use of placebo or a no treatment control group is only ethical when there exists no effective treatment. The Declaration of Helsinki 3 is clear in enunciating this distinction:

> The benefits, risks, burdens and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic methods. This does not exclude the use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic or therapeutic method exists.

In this day and age, very few diseases or illnesses do not receive at least partial benefit from standard care, therefore the use of placebo or a no treatment control could be considered as ethically dubious.

Conversely, it could also be argued that there is insufficient evidence if a placebo control is omitted from the trial. The omission could inhibit development and reduce therapeutic options.18 A recent study looked at the whether the outcomes of randomised control trials were influenced by the inclusion of a placebo group.19 A systematic review of nonsteroidal anti-inflammatory drug trials for arthritis treatment was done to evaluate the ratings of the efficacy of an active drug and the reporting of its adverse effects. They concluded, ‘if efficacy is the outcome of interest, placebo control trials may be the most appropriate study design because patients participating in placebo drug trials are more conservative in rating a drug as being effective’. Although not directly applicable to acupuncture research, is does highlight the influence that the inclusion of a placebo control can have on the subject’s belief and expectations concerning the efficacy of the treatment.

The second issue relates to impact that the delay or denial of treatment would have on the disease or illness being evaluated. Researchers are obliged to consider wheather the benefits, risks and burdens to the subject from delaying or denying treatment, outweigh the research objectives. The Declaration of Helsinki3 is again quite clear in expressing this view.

> Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others…
>
> Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits

If the disease to be treated in the trial was chronic and stable, the ethical considerations would be minimal. Control options would consist of no treatment, placebo controls or biomedical care models. As stated above, one strategy to ensure the subjects were not denied standard treatment, especially if the acupuncture was shown to be effective, is to offer the subject acupuncture treatment at the completion of the trial. This strategy again is acknowledged within the Declaration of Helsinki 3 which states that: ‘at the conclusion of the study, every patient entered into the study should be assured of access to the best proven prophylactic, diagnostic and therapeutic methods identified by the study’.

If on the other hand, the disease was progressing quickly or had serious consequences if not treated immediately, then the only ethical option would be to use the acupuncture plus standard care versus standard care only model. The delay or denial of standard treatment has to be balanced against the possible outcome of the research and how the delay or denial will impact upon the subject’s disease or illness. The principles of beneficence and maleficence have to be balanced against each other.

As has been demonstrated there is a close relationship between the choice of control, ethical issues and the need to develop a research design that is both rigorous and ethical. These three factors need to be considered early so as to develop a rigorous and ethical research model.

6. Informed consent

Obtaining informed consent from a subject prior to participating in a clinical trial is an important ethical requirement. The Declaration of Helsinki3 states that the researcher should fully inform the patient about aspects of care related to the research.

> each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely given informed consent, preferably in writing.

Informed consent safeguards the subject's 'right to be respected as a person and to have her personal goals and values given due weight by involving her in shared decision-making'.20 The inclusion of informed consent may however influence the result of a clinical trial by increasing the duration of a trial, modifying the characteristics of the population included in the trial (selection bias) and affecting the therapeutic response.21 A recent study investigated how the informed consent could be improved to reflect the patients' concerns and information needs improving enrolment and therefore minimising selection bias.22 They found that subjects often wanted information that was not ordinarily anticipated in the informed consent process. The additional use of information sheets, that incorporate specific information related to acupuncture, may well enhance subject recruitment by anticipating the concerns and information needs of potential subjects. Information such as attendance frequency, previous results from phase I or similar studies, specific side effects, contingency treatment plans and the offer of a debriefing after completion of the trial may facilitate recruitment.

7. Dissemination of results

Dissemination of research is often an overlooked ethical issue. Often clinical research that has had a negative outcome is not submitted for publication. In addition, journal editors may have a bias against publication of a negative outcome trial. Non reporting of negative trials should be seen as unethical and has been blamed for distorting the medical literature. Again the declaration of Helsinki3 is explicit in this regard stating that

> Both authors and publishers have ethical obligations. In publication of the results of research, the investigators are obliged to preserve the accuracy of the results. Negative as well as positive results should be published or otherwise publicly available.

Furthermore, acupuncture researchers may be reluctant to submit to a peer-reviewed journal because of the suggestion of reviewer bias concerning the publication of unconventional or alternative therapies. In a recent study Resch et al.23 demonstrated that there was reviewer bias concerning a fictitious but methodologically sound report on the effect of a homeopathic treatment for obesity. This is in contrast to an earlier similar study by the same research team where they found no such bias.24 Despite these equivocal outcomes and the possibility of rejection, researchers should be supported in their attempt to submit complementary and alternative medicine research for publication.

8. Conclusion

The consideration of ethics in the design and conduct of acupuncture clinical trials is fundamental to good research. Ethics must be seen as a tool that can be used flexibly. There are no definitive or final solutions to ethical issues. In fact, what the consideration of ethics does offer is a means to reflect on, and clarify issues and to see the issues in context of the research model. It is hoped that the discussion in this paper will generate more questions on the role of ethics in acupuncture research methodology.

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Breast Lymphatic Mapping and Sentinel Lymph Node Biopsy: State of the Art: 2015

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Abstract. Lymphatic mapping with sentinel lymph node biopsy (SLNB) was introduced in the 1990s as a method to stage the nodal axilla in women with breast cancer. Very quickly the technique became the standard of care because pathologic staging was more accurate and sensitive and the surgical procedure resulted in low morbidity. SLNB has continued to evolve, and the applications in breast cancer have been expanded. A review of the published data was performed to update the lymphatic mapping technique and identify key issues and trends in the application of SLNB in women with breast cancer in 2015. The importance of axillary staging continues to effect the surgical treatment of patients with breast cancer. Originally described for patients with invasive cancer, the technique now plays an important role in staging women with ductal carcinoma in situ or recurrent breast cancer and patients with advanced breast cancer who are receiving neoadjuvant chemotherapy. Histologic examinations have incorporated multiple sectioning and immunostains. The morbidity has been low, and techniques for limiting lymphedema are being introduced. Lymphatic mapping will continue to play an important role in the treatment of women with breast cancer. The SLNB will evolve by eliminating the need for radioactivity in the operating room, and the technique will become more accurate and used in expanded indications by incorporating preoperative imaging and intraoperative guidance procedures.

Keywords: Breast cancer treatment and survival; Lymphatic mapping; Micrometastases; Nodal staging; Sentinel lymph node biopsy.

1. Introduction

Axillary lymph node status is the most important prognostic factor for recurrence and survival in women with early-stage breast cancer. The lymphatic mapping technique and sentinel lymph node (SLN) biopsy (SLNB) is the reference standard for staging the axilla in clinically lymph node-negative patients. It is a procedure with low morbidity and has been proved to be safe, dependable, and reproducible for nodal staging. The SLN procedure is based on the early work by Cabanas,1 who showed that tumor cells from solid malignancies migrate in a sequential fashion through the lymphatic channels to the initial nodes in the regional basin, connected by afferent lymphatic channels. Morton et al2 from the University of California, Los Angeles, and the John Wayne Cancer Institute described the use of intradermal isosulfan blue dye injection for lymphatic mapping and SLNB in patients with melanoma in the early 1990s. Shortly thereafter, the technique was applied for nodal staging in women with breast cancer. Radioguided surgery for breast cancer was introduce by Krag et al,3 Morton and Giuliano, Norman et al,4 Reintgen et al,5 and Albertini et al.6 Norman et al4 initially showed in patients with melanoma in 1989 that preoperative lymphoscintigraphy can provide a road map for the surgeon to direct the nodal dissection. Later, Reintgen et al5 and Albertini et al6 emphasized the "orderly progression of nodal metastases" and the application of the lymphatic mapping technique to breast cancer. Previous studies have demonstrated SLN identification rates of 66% to 99%, with false-negative rates of 0% to 15% and an accuracy of 95% to 100%.7, 8 and 9 The SLN procedure allows the pathologist to perform a more detailed examination of the SLN, including more sectioning and incorporating immunostains. With these

refinements, the staging of melanoma and breast cancer became more sensitive and accurate. Combined with the low morbidity of the lymphatic mapping procedure, the technique very quickly became the standard of care for nodal staging throughout the world.

2. Relevancy of Axillary Lymph Nodes

The appreciation of the axilla as the most common site of metastatic disease for patients with breast cancer has been recognized since Wilhelm Fabry (1560-1634) first described axillary nodal excision in concert with primary tumor excision.10 What has eluded investigators since then has been understanding what the presence of axillary metastases really portends for the prognosis of the patient. In the 18th century, breast cancer progression was envisioned as an orderly process beginning in the breast and spreading to regional nodal areas before systemic spread. Halstead11 was the leading proponent of the radical mastectomy that involved en bloc resection of the breast, underlying musculature, and axillary nodes. The Halstead radical mastectomy dominated surgical treatment in the 19th century. In the early 1960s, the view of axillary lymph node dissection (ALND) being standard was challenged by Devitt12 and others when they noted that retrospective data failed to show a survival advantage for radical nodal surgery. Also, the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-04 trial13 confirmed in a prospective study that the addition of ALND to mastectomy did not improve distant disease-free survival (DFS) or overall survival (OS). The chief investigator for the NSABP trial, Bernard Fisher, in 1980 asserted that “breast cancer is a systemic disease, likely at its inception” and “the positive axillary node is a reflection of the interrelationship that permits the development of metastases rather than the instigator of distant disease.” Thus, identifying lymph node metastases is a simple method to identify tumor that has the phenotype to survive outside the breast in a host that is compromised and cannot prevent it. More recent studies have shown that tumor subtype (triple negative) does not independently predict nodal metastases; this finding reinforces the idea that patients can have a poor prognosis and be node negative.14 These triple-negative, basal genotype breast cancers have been associated with poor survival but with a lower rate of lymph node metastases than estrogen receptor-positive breast cancers. Triple-negative tumors possess the ability to disseminate and thrive in distant organs, but some feature of the tumor–host relationship prevents the development of nodal metastases.14

Although imperfect at best, the axillary lymph node continues to serve as an in vivo marker for determining whether tumor cells have gained access to the circulation and whether the cancer cells can survive outside the breast. Some believe that when cancer cells are found in the SLN, almost certainly they have gained access to the systemic circulation by the presence of lymphovenous shunts that are necessary to maintain low pressure and avoid lymphedema. Reaching the circulation might be a small task for a cancer cell; however, the ability to implant and grow in a distant organ requires a unique genotype and a cooperative host environment. The metastatic burden in the axillary node informs about the interaction between the tumor and host beyond what the primary tumor variables will provide. However, SLN metastasis has failed as a biomarker and is ineffective in predicting which treatments will be most successful. The status of the axillary lymph node remains a powerful predictor of recurrence and survival in the patients with breast cancer; however, it has some limitations, in particular, with the triple-negative, basal subtype of breast cancer, which results in a poor prognosis despite being more often node negative.

In fact, the distribution of axillary metastases between the SLN and non-SLNs might be an important driver of prognosis. Reintgen et al15 showed that, in patients with melanoma, the number of metastatic nodes, such as the current staging system suggests, is not nearly as important as whether the metastatic disease in the regional basin has made its way through the SLN to involve higher echelon nodes in the basin. In comparing patients with 2 positive nodes, if all the disease was confined to the SLNs, those patients will do much better than if the metastatic disease involves a SLN and a non-SLN. The SLN acts like a trap in the regional basin, and the primary site must shed a large number of metastatic cells to overwhelm the SLN and involve higher echelon nodes in the basin. Additional evidence that the SLN serves an important role in the regional basin is that if any metastatic disease is present, 70% of the time in breast cancer and 85% of the time in melanoma, the

disease will be confined to the SLN. By far the most common finding is involvement of 1 microscopically positive SLN. Similar studies are ongoing of women with breast cancer to ascertain the importance of regional basin metastasis distribution.

3. Ultrastaging of Cancer

As nodal staging techniques for breast cancer become more accurate and sensitive, it is apparent that the patients found to be node negative in the previous 20 years using the lymphatic mapping techniques to define the node-negative population will have better survival than the node-negative population from the 1960s and 1970s. This can be attributed to the use of lymphatic mapping to identify the SLN draining the primary tumor site. The SLN can then be more closely examined for disease using more sectioning and special stains, leading to a lower false-negative rate. Thus, patients currently considered to have stage N0 using the lymphatic mapping technique are more likely to be truly node negative and will have significantly better survival than patients considered to be node negative from the 1960s and 1970s. An ancillary benefit for this more accurately staged node-negative population is that some patients will avoid systemic chemotherapy.

Lymphatic mapping was first performed in patients with melanoma in the 1990s after a number of decades of patients undergoing complete elective nodal dissection for nodal staging. In these procedures, usually 15 to 20 nodes will be removed with radical resection and each node stained with routine hematoxylin and eosin stains of the central cross section of the node. Thus, pathologists will examine < 1% of the submitted material, and low-volume disease could be missed. With the SLNB procedure, the pathologist receives 1 to 2 SLNs and can perform multiple sections of each node, using special immunostains to help identify low-volume disease. The sensitivity of the routine histologic examination has been estimated to allow identification of 1 abnormal cancer cell in a background of 106 normal lymphocytes in complete nodal dissection specimens. With the SLNB, 1 abnormal cancer cell can be identified in 107 normal lymphocytes, an order of magnitude greater in sensitivity. The survival rates for patients with breast cancer have reflected this more accurate staging accomplished with lymphatic mapping. The 10-year survival of the node-negative population in the 1970s and 1980s with axillary node dissection as the staging procedure was 80%; however, this has increased to 90% when the lymph nodes were staged with lymphatic mapping. The same phenomenon has been found in patients with melanoma16 and patients with colon cancer.17

With lymphatic mapping and a more detailed examination of the SLN, lower volume disease has been found. The question has been debated whether isolated tumor cells [pN0(i+)] and micrometastases (pN1mi) have clinical relevance. A study by Boughey et al,18 from MD Anderson and the American College of Surgeons Oncology Group (ACOSOG) Z10 trial were analyzed. In both groups, modest, but nonsignificant, differences in DFS and OS were found, and the investigators concluded that no prognostic difference was present between women with these 2 stages of minimally identified disease and that the stage groupings should be reconsidered. The study was complicated because a large percentage of the patients in both cohorts had received adjuvant chemotherapy. Others have shown different results. An Italian study analyzed the prognostic value of the pN0(i+) and pN1mi status in a consecutive series of 702 patients from a single institution.19 By performing a more detailed histologic examination, 13% of the node-negative population was upstaged to having isolated tumor cells or micrometastases. The hazard ratio (HR) for disease relapse in the upstaged population was 2.16 ($P < .001$), and this group was shown to account for 50% of the metastatic recurrences. The Micrometastases and Isolated Tumor Cells Relevant and Robust or Rubbish (MIRROR) study, with a mean follow-up period of 5.1 years for 3181 patients, showed that systemic therapy could erase the added risk associated with the micrometastases group. 20 In addition the NSABP B-32 trial detected occult metastases in 15.9% of 3887 patients, and the presence of occult metastases was shown to be an independent prognostic variable that led to a 1.2% reduction in OS at 5 years.21 The Surveillance, Epidemiology, and End Results (SEER) database was also examined for the prognostic significance of patients with breast cancer and low-volume disease in their regional basin, and the analysis found micrometastases to be important.22 The stage 1B group remains 1 of the

key parameters of response to systemic therapy,22 and arguments with these data have been made of the importance of the detailed pathologic examination of the SLN.

4. Clinical Significance of Extracapsular Invasion of SLN

After lymphatic mapping and SLNB, a certain number of patients will be identified with extracapsular invasion (ECI) in the SLN (Figure 1). ECI can be regarded as a marker of tumor migration and invasion potential. The clinical significance of this finding is unclear, and it is questionable whether the ACOSOG Z11 study results can be applied to this population such that they can avoid ALND. A study from Japan evaluated 131 consecutive SLN-positive patients who had undergone ALND from 2003 to 2008 with regard to their long-term prognosis and non-SLN metastases.23 Of the 131 patients, 46 (35%) tested positive for ECI in their SLN. Of these 46 patients, 61% had non-SLN metastases compared with 28% of the ECI-negative group ($P < .001$). Multivariate analysis showed that ECI in the SLN evaluation is a significant predictor of non-SLN metastases (HR, 3.2; $P = .005$). The 5-year DFS rate was 71.3% in the ECI-positive group and 89.9% in the ECI-negative group ($P = .001$). Cox regression analysis showed that ECI at SLNB independently predicted for lower DFS (HR, 4.5; $P = .002$). The investigators concluded that ECI in the SLN histologic findings is an independent predictor of both non-SLN metastases and poor prognosis for patients with breast cancer.

In a review of the published data concerning this topic, ECI in the SLN was associated with higher echelon nodes in the basin involved with metastatic disease, an increased rate of both axillary and systemic recurrence, and decreased survival.24 The definitive trial supporting the elimination of complete ALND in women with a positive SLN (ACOSOG Z11 study) was a practice changing trial, and the findings of that study were used to change the standard of care in treating women with breast cancer. Patients found to have ECI in their SLNs were not eligible for enrollment. Enough published evidence is available to suggest that these women will have a greater recurrence rate both in their regional basin and systemically, such that including complete ALND should still be considered the standard of care.

5 . Technical Issues of Lymphatic Mapping

5.1. Lymph Node Mapping Agents

Krag25 initially identified the SLN in breast cancer using technetium-99m (99mTc)–labelled sulfur colloid, and this was subsequently confirmed by Giuliano et al,26 using isosulfan (Lymphazurin) blue dye, with identification rates of 82% and 66%, respectively. Albertini et al6 combined these modalities, and the identification rate increased to 92%, suggesting that the use of dual localization rates were superior to single mapping agents (Figure 2). Studies performed later in the learning curve of surgeons have demonstrated that perhaps a single agent is all that is needed. A prospective randomized trial was performed by investigators from St. Vincent Healthcare Group in Dublin, Ireland, comparing the combination of radioisotope and blue dye versus radioisotope alone in 667 patients with clinically and radiologically node-negative breast cancer.27 A total of 342 patients received the combination mapping agents and 325 patients received the radioisotope alone. Their mean age was 48 years, and the mean tumor size was 24.2 mm.27 No statistically significant difference was found between the 2 groups in tumor grade, SLN identification rate, or number of lymph nodes retrieved between the 2 groups. Also, no difference was found in the number of positive lymph nodes identified in the study (23.8% vs. 22.1%; $P = .64$). 27 The study failed to demonstrate an advantage with the addition of isosulfan blue dye to radioisotope in the identification and harvesting of SLNs, as long as SLNs were visible on the preoperative lymphoscintigram (Figure 3). A meta-analysis, which examined 69 trials and > 8000 patients, seemed to confirm these findings.28 The identification rates for blue dye alone (19 studies), radiocolloid alone (16 studies), and a combination of the 2 mapping agents (34 studies) were 83.1%, 89.2%, and 91.9%, respectively. The corresponding false-negative rates were 10.9%, 8.8%, and 7%.28 The investigators concluded that although the increase in the identification rate with the combination blue dye and radiocolloid was

slight, this was not an independent predictor of false-negative SLNB rates on multivariate analysis, and, therefore, it would be acceptable to use either technique alone.28

Radiation exposure and the disposal of contamination in the surgical suite are 2 issues that have not been solved. More recent studies have reported that vital blue dye can be eliminated, which is reasonable, considering the small, albeit real, incidence of significant anaphylaxis.29 Also, one can avoid skin tattooing and any interference with pulse oximetry, as long as the preoperative lymphoscintigram shows a strong unique signal in the axilla, separable from injection site radioactivity. However, many institutions have eliminated imaging after radiocolloid injection, because surgeons will only be performing lymphatic mapping to the axilla. Although it might be helpful for identifying intramammary lymph nodes in the upper outer quadrant and keying the surgeon that these might be present, intraoperative scanning with the gamma probes should be able to find the intramammary nodes. Thus, if one is only concerned with axillary mapping, imaging becomes less useful. This policy decreases costs and still provides accurate lymphatic mapping to the axilla. However, the Holy Grail in lymphatic mapping would be to eliminate radiocolloid from the operating room.

The current reference standard for the detection and targeted excision of the SLN is preoperative lymphoscintigraphy with 99mTc (Figure 3). Because surgeons are most concerned with performing accurate mapping to the axilla, the radiocolloid is injected in the ipsilateral subareolar plexus, and the time and expense of imaging is not recorded. However, there is a worldwide shortage of 99mTc; thus, alternative nonradioactive dyes for SLN labeling must be found. Indocyanine green (ICG) has been considered a possible alternative. A prospective clinical trial was performed to compare the usefulness of ICG versus 99mTc for the identification of SLNs.30 The preoperative and intraoperative SLN detection rates were compared. The study showed that SLN location was identified in all cases before surgery using 99mTc; however, visualization with ICG green before the skin incision was only possible in 17 of 80 patients (21%).30 However, SLN identification using the near infrared fluorescence technique in the operative site after skin incision and initial tissue preparation was 141 of 147 (96%), making it comparable to 99mTc. Although using ICG eliminates the need for handling any radioactive material and would be a major advantage, the new marker does not perform up to the reference standard, 99mTc, in preoperatively identifying all nodal basins at risk of metastases and providing the surgeon with the information needed to perform the dissection. This quality is less important in breast mapping, because the surgeon is most concerned with accurate mapping to the axilla. ICG has been used successfully in performing lymphatic mapping for gynecologic malignancies, with marked improvement of bilateral SLN detection rates of 96% versus 61% compared with dye and radiocolloid.31

Another novel mapping agent, [99mTc]tilmanocept, a new CD206 receptor-targeted radiopharmaceutical agent, was evaluated for its use in lymphatic mapping in a series of patients with intraoral or cutaneous head and neck cancer undergoing primary tumor resection, SLNB followed by complete lymph node dissection (CLND).32 All patients were considered clinically node negative at the time of the study. The mapping agents in use at that time, radiocolloid and vital blue dye, are characterized by a nonspecific accumulation of the agents in the SLN by macrophages and dendritic cells. The small molecular size (7-nm diameter) of tilmanocept and its specific targeting to CD206 mannose-binding receptors located on reticuloendothelial cells within the lymph node permit rapid injection site clearance and avid, stable binding within the target nodes.32 Tilmanocept identified ⩾ 1 SLNs in 81 of 83 patients (97.6%). Of the 39 patients with tumor-positive regional nodes, 1 patient had a single tumor-positive non-SLN, for whom all SLNs were tumor negative, for a false-negative rate of 2.6%. The negative predictive value was 97.8%, and the overall accuracy was 98.8%.32 No differences were noted between the same-day and next-day mapping procedures. Compared with SLN mapping of head and neck cancer in published studies using blue dye and radiocolloid (false negative rate, 10%), the false-negative SLN rate appeared to be improved and could be used in this population to obviate the need for elective lymph node dissection. The specificity of tilmanocept for lymphatic tissues assessed by in vivo imaging and in vitro analysis of its receptor binding properties suggest that tilmanocept does not move downstream to distal second station lymph nodes, permitting

high confidence that the hot node found during next-day procedures will be the SLN. The ability to perform the mapping injection the day before surgery without any decrease in accuracy provides flexibility in scheduling. Recently, a study using this compound was performed in women with breast cancer.33 A total of 13 centers enrolled 148 patients, who were injected with both tilmanocept and vital blue dye. Intraoperatively, 207 of 209 nodes detected by blue dye were also detected with tilmanocept, for a concordance rate of > 99%. Of the 33 pathology-positive nodes (18.2% patient-positive pathology rate), tilmanocept detected 31 of 33 compared with 25 of 33 for blue dye (P = .03). The investigators concluded that tilmanocept identified more SLNs in more patients and a higher number of metastatic breast cancer lymph nodes than identified by blue dye. 33

The cost of technetium sulfur colloid has increased recently, and other agents are now being studied in attempts to find better specificity and eliminate radioactivity from the operating room. However, until improvements are realized, technetium sulfur colloid will remain the reference standard.

5.2. Site of Mapping Agent Injection

Multiple different sites of tracer injection have been used, with the easiest and most effective location the subareolar plexus of Sappey.34 This site has proved to be superior to other injection sites, because a high percentage of the injectate reaches the axillary more quickly as it follows the natural progression of lymph flow from the subareolar plexus. Combined with a reduction in "shine through" from injecting around tumors in the breast parenchyma located in the upper, outer quadrant and the fact that the cancer does not have to be located in the breast, the subareolar injection has proved to be the preferred injection site for breast lymphatic mapping, with the caveat that it will not light up any internal mammary (IM) lymph nodes. Most groups will not harvest any IM nodes found on preoperative lymphoscintigraphy studies; thus, this might not be an important limitation.

5.3. Intraoperative Examination of SLN

One approach in an attempt to gain intraoperative information on the status of the SLN is to perform frozen section analysis of the SLN. However, the low-volume disease in the SLN and the waste of valuable material as the sections are cut on the cryostat are significant shortcomings. Touch preparation techniques performed directly on the SLN avoids the waste of material with the cryostat; however, institutions must have good cytology interpretation for effective use. Others will submit fresh, nonfixed SLNs to the pathology laboratory for macroscopic analysis, with frozen section analysis performed on grossly suspicious SLNs.35 If positive, the surgeon has the option of completing the ALND. If negative, the SLNs are fixed, paraffin-embedded, and sectioned at 2-mm intervals for routine and immunostain examination.

6. Extra-Axillary Sites of Lymphatic Flow

Preoperative lymphoscintigraphy in women with breast cancer has imaged drainage to the SLNs in the axilla (Figure 3); however, in approximately 10% of patients, drainage has also been seen to extra-axillary sites, most commonly the IM nodes and the subclavian or ipsilateral neck nodes. These patients can be identified with preoperative lymphoscintigraphy studies if the radiocolloid is injected into the breast parenchyma around the primary tumor. The lymphatic channels that lead to the IM basin initially go deep through the pectoralis fascia to the IM chain. Injections into the skin above the tumor or the subareolar plexus will not image these extranodal sites.

The question remains regarding the clinical relevance of this multidirectional drainage because most patients will also receive adjuvant chemotherapy and/or hormonal therapy, and a percentage will also undergo adjuvant radiation therapy. A recent study evaluated the incidence and prognostic effect of metastatic IM SLNs.36 During a 13-year period, 3685 patients underwent breast surgery and SLNB after intratumor or peritumoral injection of radiocolloid. In 754 patients (20.5%), ipsilateral IM SLNs were visualized on preoperative lymphoscintigraphy. The harvest rate of IM SLNs was 81%. IM metastases were detected in 21.3% of the harvested SLNs and 3.5% of all patients. The presence of IM metastases was associated with axillary metastases (P < .001). With a mean follow-up period of 61 months, 10.9% of the patients had died. A multivariate analysis showed that IM

metastases did not have a significant effect on overall survival unless the patients had IM metastases alone without axillary metastases.

Most groups have ignored the IM chain or IM lymphatic flow found on preoperative lymphoscintigraphy studies because harvesting this site is technically demanding, adds another scar to the patient undergoing lumpectomy to treat her primary tumor, and the clinical relevance is uncertain because most patients will also receive total body therapies in the form chemotherapy and/or hormonal therapy and might also receive adjuvant radiation therapy to this area. Most US surgeons have confined their energy to the performance of accurate axillary SLN mapping.

7. Predicting Extent of Nodal Metastases—The Promise of Axillary Imaging

Enhanced axillary imaging is an area of active investigation as an approach toward more accurate preoperative nodal staging.37 These procedures can possibly, not only save node-negative patients from undergoing unnecessary axillary surgery, but could also help to distinguish node-positive patients who might be treated appropriately with SLN resection alone from those who would benefit from more extensive ALND. Axillary ultrasound (AUS) can be used to identify suspicious nodes, followed by ultrasound-guided fine needle aspiration (FNA). In a study by Caudle et al,38 708 patients with node-positive T1 and T2 invasive breast cancer evaluated from 2002 to 2012 underwent surgery directly after diagnosis and did not undergo neoadjuvant chemotherapy (NAC). These patients were stratified according to whether their node-positive disease was identified by preoperative AUS and FNA (190 patients) or by SLN resection (518 patients). The investigators found that those diagnosed as node-positive by preoperative AUS and FNA were substantially more likely to have ⩾ 3 metastatic axillary nodes, larger nodal metastases, and extranodal disease extension compared with those deemed node-negative after AUS and FNA.38 Furthermore, the study identified significant axillary disease burden in patients with 1 to 2 suspicious lymph nodes found by AUS and a positive preoperative lymph node on FNA. In addition, the presence of infiltrating lobular histologic features, but no other clinicopathologic features, was associated with ⩾ 3 positive nodes at surgery. Finally, their study found that ⩾ 3 suspicious lymph nodes found by AUS among the FNA-positive patient group was associated with pathologic stage N2 or higher disease in 60% of patients.38 From these findings, Caudle et al38 concluded that AUS and FNA are useful in predicting the nodal disease burden and suggested caution in the omission of ALND for AUS-detected patients, who might not be comparable to SLN resection-detected patients in the ACOSOG Z0011 trial.37 and 38 AUS can be used as a tool to select patients with a high axillary disease burden who are likely to benefit from ALND and other more aggressive therapies.

8. Morbidity of SLNB and Avoidance of Lymphedema

The ALMANAC trial39 (Axillary Lymphatic Mapping Against Nodal Axillary Clearance) studied the morbidity associated with lymphatic mapping and demonstrated that SLNB is associated with a significant reduction in overall morbidity. This group from the United Kingdom conducted a multicenter randomized trial to compare the quality of life outcomes between patients with clinically node-negative breast cancer who had undergone SLN versus those who had undergone CLND of the axilla. A total of 1031 patients were randomly assigned to 1 of the 2 axillary procedures. Patients with SLN metastases underwent CLND or axillary radiotherapy. The relative risks of any lymphedema or sensory loss for the SLNB group compared with standard CLND of the axilla at 12 months was 5% versus 13% and 11% versus 31%, respectively. Drain usage, length of hospital stay, and time to resumption of normal day-to-day activities after surgery were statistically significantly lower in the SLNB group ($P < .001$), and the operative time was reduced ($P = .05$). Patient-recorded quality of life and arm functioning scores were significantly better statistically in the SLN group throughout all periods tested (1-12 months). These benefits were seen without any increase in anxiety level in the SLN group.

Lymphedema in breast cancer patients causes a long-term decrease in quality of life, as well as chronic pain, depression, and anxiety (Figure 4). The percentage of patients with breast cancer experiencing lymphedema after undergoing ALND has ranged from 20% to 45% and increases if the patient also receives adjuvant nodal radiotherapy.40 The significant effect on quality of life and the requirement for lifelong therapy demands that effective preventative strategies be investigated. Lymphatic mapping and SLNB resulted in the potential to avoid this complication by just removing the 1 to 2 nodes most likely to contain metastases. However, even in the best of hands, a small, albeit real, chance (1%-3%) exists of lymphedema developing after SLNB. The factors shown to increase the risk of secondary lymphedema include the number of nodes dissected, the use of extended nodal radiotherapy, and body mass index > 30 kg/m2.41 Current management involves symptom relief with manual lymph drainage with massage, compression garments, and physical therapy; however, this requires extended treatment with a complaint patient.42 Breast cancer survivors with lymphedema report long-term morbidity that includes chronic pain, depression, and anxiety. The medical costs are high and the loss of work productivity is significant.43

Axillary reverse mapping is a technique developed by Klimberg44 and others in an attempt to eliminate this complication. This procedure is performed in conjunction with the lymphatic mapping procedure. Radiocolloid is injected into the subareolar plexus for axillary lymphatic mapping, and blue vital dye is injected into the proximal ipsilateral, medial arm to identify the arm lymphatics entering the axilla. With axillary reverse mapping, an attempt is made to spare all lymphatics coming from the arm while harvesting the SLN. If the breast SLN is the same as a node receiving a blue lymphatic from the arm, the SLN is harvested, and some form of a lymphatic/venous anastomosis is performed. This technique has resulted in a decreased in the lymphedema rate after SLNB.

For patients with grossly positive nodes or women with a positive SLN after mastectomy, CLND is recommended. Techniques are currently being developed to avoid lymphedema even with this more radical procedure. The lymphatic microsurgical preventive healing approach (LYMPHA)40 for the primary prevention of lymphedema is one such procedure. Originally described by Boccardo et al45 in 2009, they reported a 4.05% rate of ongoing lymphedema in a population of 74 patients who had undergone axillary dissection with a 4-year follow-up period. Afferent lymphatic channels, identified by the injection of vital blue dye in the ipsilateral upper arm, that have been divided by the axillary dissection procedure are sutured into a branch of the axillary vein distal to a competent valve. The site of the anastomosis is necessary to prevent clotting. Pre- and postoperative lymphoscintigraphy that includes arm measurements and bioimpedence spectroscopy are performed. In a 2015 series from Columbia University, Feldman et al,40 performed LYMPHA in 37 women who were undergoing ALND during a 26-month period. Successful completion of the anastomosis occurred in 27 women (73%), with an average size of the lymphatic channels of 1 to 2 mm. The unsuccessful attempts resulted from the lack of a suitable vein, lack of a suitable lymphatic, or extensive axillary disease. The mean follow-up period was 6 months. The body mass index was > 30 kg/m2 in 37% of the women, and 63% had received axillary radiotherapy. The lymphedema rate was 12.5% in the 24 patients with a successfully completed anastomosis and 50% in the 8 unsuccessfully treated patients. No LYMPHA-related complications occurred. Comparing patients with completed and incomplete LYMPHA with ⩾ 3 months of follow-up, the odds ratio for the development of lymphedema with LYMPHA versus no LYMPHA was 0.14 (95% confidence interval, 0.02-0.90, with a Fisher exact probability test for 2-tailed P = .05). These early data from a high-risk cohort of patients suggests that LYMPHA is feasible, safe, and effective as a method for the primary prevention of clinical lymphedema. 40

9. SLNB in Patients With Ductal Carcinoma In Situ

The incidence of ductal carcinoma in situ (DCIS) has increased dramatically in the United States and other countries with the proliferation of breast cancer screening programs. Approximately 20% of total breast cancer cases will be DCIS, and 5% to 13% of these patients will have microinvasion of the tumor cells into the surrounding stroma.46 Microinvasion is defined as a ⩽ 1.0-mm extension of

tumor cells into the surrounding stroma. Furthermore, the pathologist might report findings such as "suspicious for microinvasion" or "microinvasion cannot be excluded" when tumor cell nests or single cells appear to be focally extending outside a pre-existing ductal lobular structure in a background of high-grade DCIS.46 The proper identification of microinvasion in DCIS is of high importance because the presence of microinvasion could dictate performing SLNB to evaluate the axilla for regional metastasis. For pure DCIS (no microinvasion), SLNB is not recommended, except in cases with a suspicious mass on imaging or a large area (⩾ 5 cm) of calcifications without a mass. Additional factors associated with a greater risk of invasive breast cancer and subsequent nodal disease in the context of DCIS include a palpable mass, multicentric disease, high nuclear grade, necrosis, use of smaller gauge biopsy needles, and a core needle biopsy reported as DCIS with findings suspicious for microinvasion. The rate of upstaging can be as great as 91% for invasive cancer in patients with 4 of these high-risk characteristics on core needle biopsy, and DCIS with 1 high-risk characteristic has been associated with a 12% rate of SLN involvement, although > 75% of those were micrometastases. At present, SLNB is the standard of care for all invasive breast cancers. However, in a retrospective study performed by Namm et al,46 about 66% of patients with findings suspicious for microinvasion without upstaging to invasive disease could have been spared the potential morbidity of SLNB if they had not been offered SLNB until after a definitive diagnosis of invasive cancer was made. Therefore, these researchers concluded that until clinically significant lymphatic invasion can be better predicted, surgeons should consider omitting SLNB in low-risk cases (those without findings suspicious for microinvasion) until invasive ductal carcinoma has been confirmed by surgical resection to prevent the morbidity of SLNB for most patients.

However, these treatment guidelines do not address the situation of women diagnosed with DCIS on image-guided core biopsy and electing to undergo mastectomy. In these cases, if invasive cancer is found on mastectomy, nodal staging cannot be performed and lymphatic mapping cannot be offered. In a multicenter study from France, investigators sought to determine the benefit of performing upfront SLNB in these women.35 The secondary aim of their study was to determine the pathologic variables associated with finding microinvasion or invasion in the mastectomy specimen. From 2008 to 2010, 228 patients were enrolled from 14 French cancer centers, including 192 patients with pure DCIS on biopsy. The mammographic findings were either extensive microcalcifications or multicentric foci (in 2 different quadrants of the breast). ALND was avoided for 67% of the patients with microinvasive DCIS or DCIS associated with invasive breast cancer at mastectomy and found to have a negative SLN. Of the 192 patients with pure DCIS on biopsy, 39% were upgraded to invasive cancer after mastectomy. This rate was greater than other published series47 and might have resulted from the large size of the DCIS lesions in the series (mean size, 69.3 mm). The rate of positive SLNs for patients with pure DCIS on biopsy was 14%. High nuclear grade and human epidermal growth factor receptor 2/neu-amplified DCIS was associated with a greater risk of finding invasive cancer after mastectomy. The investigators concluded that underestimation of invasive components is high when DCIS is diagnosed by biopsy. Upfront SLNB for patients with extensive DCIS avoids unnecessary ALND for two thirds of patients with invasive disease found only after mastectomy.

A recent study addressed the clinical relevance of positive SLNs in women with DCIS.48 That report identified 1234 patients from a single institution with an initial diagnosis of DCIS who had undergone SLNB. Positive SLNs were defined as either isolated tumor cells (⩽ 0.2 mm), micrometastases (> 0.2-2 mm), or macrometastases (> 2 mm). Positive SLNs were identified in 10.7% of the population, 66 patients with isolated tumor cells, 2.9% of patients with micrometastases, and 2.4% with macrometastases. Upstaging to microinvasive or invasive cancer occurred in 26.5% of the patients. The variables associated with a positive SLN included diagnosis by excisional biopsy, DCIS > 2 cm, > 3 interventions before the SLNB, and occult invasion. Patients with pure DCIS, independent of their SLN status, had equivalent survival that approached 100%. Patients with occult invasive cancer and positive SLNs had worse survival (91.7%). That > 3 interventions in the breast before the SLN procedure was associated with positive SLNs without an effect on survival would support the theory that benign mechanical transport of breast epithelial cells occurs with breast

manipulations. The study also concluded that except for patients at high risk of invasive disease, the routine use of lymphatic mapping for patients with DCIS is not warranted.

10. Lymphatic Mapping in Conjunction With NAC

In patients with more advanced breast cancer, NAC has been recommended in an attempt to increase the breast preservation rate without having an effect on the survival data. Although previously cases of documented node-positive disease before the advent of NAC resulted in complete ALND, the improvements in pathologic complete response rates seen with the use of targeted agents now suggest that more radical surgery might not be necessary. The ACOSOG Z1071 trial, SENTINA (sentinel-lymph-node biopsy in patients with breast cancer before and after NAC) trial in Europe49, and the Canadian Sentinel Node Biopsy Following NeoAdjuvant Chemotherapy in Biopsy Proven Node Positive Breast Cancer (SN FNAC) trial50 showed that the false-negative rate of SLNB after chemotherapy for patients presenting with node-positive disease is 8% to 14%.47, 48, 49, 50 and 51 The ACOSOG Z1071 trial reported a 12.6% false-negative rate for SLN surgery after NAC. In that study, patients with T4N1-N2M0 breast cancer underwent AUS after NAC. Post-NAC AUS images were reviewed for 611 patients, and 71.8% of the AUS-suspicious patients were node positive at surgery compared with 56.5% of the 430 AUS-normal patients. Patients with AUS-suspicious nodes had a greater number of positive nodes and a larger metastatic size ($P < .001$). However, in the setting of normal AUS findings, only 39% of the women had a complete pathologic response. The high percentage (61%) with disease remaining in the axilla after NAC and normal AUS findings underlines that some form of nodal staging remains important after NAC. AUS is operator dependent, but it has outperformed other imaging modalities such as positron emission tomography and magnetic resonance imaging. Using a strategy in which only those patients with normal AUS findings would undergo SLNB would reduce the false-negative results for SLNB from 12.6% to 9.8%. The study concluded that AUS should be recommended after chemotherapy to guide axillary surgery. A false-negative rate of 9.8% with a combination of AUS and SLNB would be acceptable for the adoption of SLNB in women with node-positive breast cancer undergoing NAC. 52 Factors that decreased the false-negative rate included the resection of $\geqslant$ 2 SLNs, the use of 2 mapping agents, the use of immunhistochemical (IHC) staining and the placement of a clip in the positive node at diagnosis with removal of the node at postchemotherapy surgery.53 Historically, patients who initially presented with clinically positive axilla would undergo CLND. Data from the ACOSOG Z1071 trial support a potential new use for AUS for the evaluation of the axilla in an increasing population of women undergoing NAC. The overall in-breast tumor response to NAC was not assessed in the trial and because residual disease in the breast indicates a poor response to chemotherapy and might indicate remaining disease in the axilla, this factor could be important in selecting women for SLNB.

Other groups have used pretreatment tattooing (sterile black carbon suspension) of biopsied axillary lymph nodes to later remove them.54 Choy et al54 showed that the tattooed nodes are visible intraoperatively months later, obviating the need for additional localization during axillary staging. The National Comprehensive Cancer Network guidelines now recommend performing the SLNB as an option for women receiving NAC. Increasingly nationwide, patients with node-positive breast cancer treated with NAC can undergo SLNB to evaluate the nodal response to chemotherapy and reserve axillary dissection for those patients with residual nodal disease. A recent report used preoperative, ultrasound-directed wire localization to improve the accuracy of axillary lymph node surgery after a previous node had been biopsied and proved to harbor metastases.55 Wire localization of the positive node or previously placed biopsy clip resulted in a 97.3% surgical removal rate compared with 79.4% if no wire localization was used and resulted in more accurate staging and a decreased false-negative rate for SLNB after neoadjuvant therapy.

11. Do Older Women With Breast Cancer Need SLNB?

Women aged > 65 years are the fastest growing subset of the American population in terms of breast cancer diagnoses and death rates, and both these factors increase with increasing age.56 Moreover, the National Cancer Institute has reported that 41.2% of newly diagnosed breast cancer cases and 57.6% of deaths occur in women aged > 65 years. A study by Sun et al56 found that older women with early-stage breast cancer were also more likely to forgo radiation and lymph node staging than their younger counterparts. This might be because of the perceived reduced benefits of radiation and lymph node staging; however, the investigators showed that forgoing these treatments was associated with a negative effect on overall survival and breast cancer-specific survival.56 After controlling with propensity score matching, patients who received radiation had a 7.4% greater survival rate, and patients who underwent lymph node staging had a 16.8% greater survival rate. For breast cancer-specific survival, the mortality rate with receipt of radiation and lymph node biopsy was 1.3% and 2.6% lower, respectively. Therefore, it is important to follow the standards of care when treating older patients, despite the perceived reduced benefits of radiation therapy and lymph node staging.56

12. SLNB for Breast Cancer Recurrence

With the widespread use of breast-conserving surgery (BCS) and the increased accuracy of diagnostic imaging techniques, the rate of ipsilateral breast tumor recurrence within 10 years after BCS has ranged from 5% to 10%.57 and 58 However, the standard treatment for these women remains controversial. In a recent update of the American Society of Clinical Oncology, the indications for lymphatic mapping were broadened to include those women with previous nononcologic axillary surgery. However, the guidelines did not address those women who had undergone previous SLNB. In cases of breast cancer involving local ipsilateral recurrences in which SLN dissection has already been performed, the standard of care is to perform ALND. The main argument against a second SLNB procedure is that the lymphatic channels have been disrupted by scarring from the initial surgery and that any postoperative radiation therapy would affect efforts to perform a second SLN procedure. However, it is possible that the use of SLN dissection can be further extended to locally recurrent breast cancers to spare the morbidities associated with ALND.59 Criticisms of this procedure include that these patients already have lymphatic pathways damaged by the first operation and possibly by adjuvant radiotherapy, which serves to lower the feasibility and accuracy of the procedure in such cases. In a study by Caspara et al,59 147 patients with locally recurrent breast cancer were examined. All patients were negative for metastatic lymph nodes on preoperative ipsilateral AUS. One half of the patient population had undergone SLN dissection and one half, ALND; 124 patients (84.4%) had previously undergone radiotherapy. Lymphoscintigraphy was performed before SLNB after breast cancer recurrence in 82% of patients—77% in the case of previous SLN dissection and 88% in the case of previous ALND. In approximately one half of these patients, a SLN was identified, and 55 of the 72 patients (76.4%) with successful SLN dissection after breast cancer recurrence were node negative. In 14 of the 17 patients with positive SLNs, metastases were located in the ipsilateral axilla, 9 of whom had undergone previous SLN dissection and 5, previous ALND. Other sites of metastases included intramuscularly in the pectoral muscle, ipsilaterally and intramammary in another, and the contralateral axilla in another patient who had undergone ALND.59 The study by Caspara et al59 has demonstrated that SLN dissection after breast cancer recurrence is a feasible procedure, with a detection rate of approximately 50%. In patients who had previously undergone SLN dissection, 37 of 73 (51%) were node negative, and these patients could thus be spared the morbidity associated with ALND. In addition, 11 patients had only micrometastases or isolated tumor cells in the SLN at recurrence. None had non-SLN involvement and thus could also be spared ALND. Eight percent of the patients had macrometastases in the SLN at recurrence compared with patients with primary breast cancer, and only 1 of these patients had non-SLN metastases and might have benefited from ALND. In 6 patients (8%), who had previously undergone ALND, the treating physicians persisted and examined the axilla for draining nodes and, when found, performed axillary dissection. Metastases were found in all 6 patients. This disease would have been overlooked if the current guidelines that state that no additional lymph nodes should

be removed if ALND has already been performed were followed. Finally, the study by Caspara et al59 reported that a substantial number of patients had drainage to aberrant SLNs, and this number was even more prevalent after previous ALND or mastectomy. Metastases in aberrant SLNs would not be removed by surgery according to the current guidelines.59

In another study addressing this issue from the European Institute of Oncology in Milan, Italy,60 212 patients with the diagnosis of operable local breast cancer recurrence were studied. All these patients had previously undergone lumpectomy and an initial negative SLNB. The results showed that preoperative lymphoscintigraphy demonstrated ⩾ 1 new SLN in 207 patients (97.7%). One or more SLNs were surgically removed from 196 of the 207 patients (92.5%). Extra-axillary drainage pathways were seen in 8%. The annual axillary recurrence rate after a median follow-up period of 48 months was 0.8%, and the cumulative incidence of axillary recurrence at 5 years was 3.9%. They concluded that a second SLNB should be considered for women with operable local breast cancer recurrence to stage the axilla after recurrence, identify extra-axillary sites of drainage, and remove all signs of local and regional spread of disease.60 They hypothesized that a postoperative collateralization of lymphatics occurs as a physiologic compensatory mechanism and that the new lymphatic pathways will allow the identification of new SLNs.60 Because the percentage of aberrant lymphatic drainage pathways outside the ipsilateral axilla in patients with previous BCS was 2.2% to 47%,60 a central role exists for preoperative lymphoscintigraphy in these patients to identify all possible routes of spread.

A recent meta-analysis of 26 studies regarding repeat SLNB for 692 patients with locally recurrent breast cancer showed high success rates for SLNB used for lymphatic mapping and identification of SLNs and acceptable identification of extra-axillary drainage for patients with previous axillary surgery.61

13. Health Care Disparities

Black women with early-stage breast cancer are significantly less likely than their white counterparts to undergo SLNB. In a study from MD Anderson Cancer Center,62 with a review of 31,274 women aged ⩾ 66 years diagnosed with early-stage breast cancer from 2002 to 2007, significantly fewer black patients than white patients underwent a SLN procedure (62% vs. 74%; $P < .001$). The SLNB rate increased in both groups during the study period, only less so in the black population. The retrospective analysis from the SEER database showed that black women were also more likely to develop lymphedema, with a 5-year risk of 12.3% for black women compared with 8.2% for white women ($P < .001$). That study was the first to demonstrate that the lower frequency use of SLN procedures in the black population had an adverse clinical outcome resulting in more lymphedema.

14. Can ALND Be Omitted in Patients With a Positive SLN?—the ACOSOG Z0010-11 Study

The ACOSOG set out in their initial clinical trial effort to establish the clinical significance of SLN and bone marrow (BM) micrometastases. A total of 5539 patients were entered in the study, with a SLN identification rate of 94.5% in the national multi-institutional study. Hematoxylin and eosin staining detected metastases in 23.9% of the patients. Using IHC staining, an additional 10.5% of patients were identified with SLN metastases. BM metastases were identified in 3% of the patients. A multivariate analysis showed that SLN or BM metastases, estrogen and progesterone receptor negativity, larger tumor size and higher grade were associated with poorer survival. IHC metastases in the SLN ($P = .66$) or BM ($P = .08$) were not independent predictors of overall survival. The study concluded that IHC examination of the SLN identified disease that might not be clinically relevant, although a strong trend was found that IHC-detected BM metastases were clinically important. 63

The ACOSOG Z0011 trial64 was a randomized trial of axillary dissection in women with clinical T1-T2N0M0 disease with a positive SLN, who had undergone lumpectomy and adjuvant radiation therapy as their primary breast cancer treatment. A total of 891 patients were randomized to observation versus CLND after a positive SLNB. With a median follow-up period of 6.2 years, no

trend was found for clinical benefit of ALND for patients with limited nodal disease (Figure 5). These findings were practice changing for surgeons and significantly changed the surgical management of the axilla. The ACOSOG Z0011 trial found that omitting ALND did not lead to inferior survival or local recurrence if the patients met the following criteria: undergoing BCS with radiation therapy, favorable low T stage, no more than 2 involved SLNs, and no gross extracapsular extension in the involved nodes. The American Society of Clinical Oncology later updated their practice guidelines to incorporate the findings of the ACOSOG Z11 trail, stating that those women who met these criteria should not undergo ALND. The results of the ACOSOG Z11 trial has resulted in a significant decline in women receiving CLND nationally. The question remains regarding the standard of care for women undergoing mastectomy and SLNB to treat their breast cancer. Because these women might not be receiving adjuvant chest wall radiation postoperatively, a treatment that probably also incorporates level 1 and 2 lymph nodes in the radiation fields, they would still be candidates for CLND after a positive SLNB.

15. Conclusion

Lymphatic mapping and SLNB will continue to play an important role in the treatment of women with breast cancer. Although some controversy exists in determining the effect of nodal staging on the treatment and prognosis of women, the knowledge gained from the technique continues to be used to guide therapy and determine the prognosis. The SLN procedure will evolve by eliminating the need for radioactivity in the operating room, and the technique will become more accurate and used in expanded indications by incorporating preoperative imaging and intraoperative procedures.

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Identification and Functional Assessment of Novel Gene Sets towards Better Understanding of Dysplasia Associated Oral Carcinogenesis

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Abstract. Oral epithelial dysplasia (OED) often precedes oral cancer. Understanding the underlying complex biological aspects of dysplasia associated oral carcinogenesis using important gene sets is thus important. Computation assisted gene set identification through different feature ranking and visualization techniques was therefore attempted in this study. Result suggested that, weighted support vector machine (SVM) could be useful for feature ranking and SVM for attribute selection. Alteration in keratinization, cell–cell communication and peptidase activity was the major affected phenomena, while extracellular matrix dynamics was also found to be hampered. During best gene subset identification, set of six genes could classify normal (NOM) and oral squamous cell carcinoma (OSCC) conditions and two sets comprising four genes in each could classify NOM and dysplastic (DYS) conditions with 100% sensitivity and specificity. A gene set, comprising 32 genes showed best efficacy of 94.12% sensitivity, 99.40% specificity and 98.89% accuracy during classification of DYS and OSCC.

Keywords: OED, oral epithelial dysplasia (OED); SVM, support vector machine; NOM, normal; OSCC, oral squamous cell carcinoma; DYS, dysplastic.

1. Introduction

Oral epithelial dysplasia (OED) is often a step that precedes development of squamous cell carcinoma. It can either convert to oral squamous cell carcinoma (OSCC) or revert back to normal condition, if treated early. Till date there are no specific biomarkers which may be precisely utilized to assess malignant potentiality of oral precancers including OED. Histopathological evaluation of biopsy specimens still serves as gold standard for critical detection of grades of dysplasia and for predicting its malignant potentiality. However, the procedure lacks specificity and suffers from inter and/or intra-observer variability because of the paucity of unequivocal features of dysplasia that may be regarded as cardinal markers for accurate prediction of progression risks in oral pre-malignant disorders A recent review suggested that combination of selected biomarkers may be effective to address such problem (Banerjee and Chatterjee, 2015).

OED is a histopathological condition, where cytological and architectural characteristics of oral mucosa are altered. The role of OED in oral carcinogenesis is quite controversial. Some literature suggests that likelihood of malignant transformation of OED is significant (Al-Dakkak, 2010), while other studies have shown that there is no correlation between malignant potentiality and grade of dysplasia (Dost et al., 2014). In such circumstances, understanding the molecular progression of OED to OSCC is important and can no longer be avoided (Pitiyage et al., 2009). Semi-quantitative analysis of immunohistochemically stained tissue sections has been attempted to grade OED in precancers, (Anura et al., 2014) however, the procedures are still immature and have not yet been utilized in routine clinical practices. Comparative and quantitative assessments of histological grading and immunohistochemical expression of few key molecules to study the association between OED and OSCC were reported in few studies (Anura et al., 2014 and Tabor et al., 2003). Molecular dissection

of oral carcinogenesis has also been attempted through the analysis of proteome and deregulation of molecular network (Molinolo et al., 2009), but understanding the progression of OED to OSCC remains in its infancy. In silico analysis of microarray gene expression data is recently gaining interest for selection of candidate gene which may be subjected to gene ontology (GO) and functional enrichment analysis for understanding underlying molecular, biological and cellular activities of given gene sets and prioritizing candidate diagnostic indicators (Hindumathi et al., 2014).

In this study, an in-depth bioinformatic and statistical analyses of the microarray transcriptome were attempted to throw light on the process. Differentially expressed (DE) genes were primarily selected to dissect progression of OSCC through OED. Weighted support vector machine (SVM) was employed to select precise gene subset towards optimal classification of oral lesions, OED and OSCC. Venn diagram was implemented in visualization of complex association of different gene sets, to unearth their possible functional association (Kestler et al., 2005). The major aim of this cost-minimized strategy exercise is to select a novel gene sub-set which can modulate specificity and sensitivity of the classification task.

The main challenge of microarray data analysis includes high number of variables against a small sample size, from which meaningful gene sets have to be chosen which should classify the disease with maximum efficiency at optimum computational burden and diagnostic cost (Liu et al., 2011). Supervised machine learning classifiers such as Naïve Bayes (NB) (Wu et al., 2012) and k nearest neighbor (KNN) (Zhang and Deng, 2007) are commonly used for cancer microarray data classification in addition to support vector machine (SVM). In this study efficiency of these three classifiers were evaluated. Feature ranking and feature selection are routinely used to reduce data dimensionality and improve learning and predictive efficiencies. A recent study showed feature ranking utilizing weights from linear SVM yields better result even with non-identically distributed training and testing data [13]. ReleifF is a feature selection algorithm, which acts through filtering and is popularly used in cancer microarray data analysis. It randomly draws instances and after computing the nearest neighbors, weighs the feature. It comparatively provides higher weightage to the attribute which have higher differentiating ability of the instance from neighbors of other class (Wang and Makedon, 2004). Efficacy of feature selection algorithms such as weighted SVM was also evaluated in this study during gene selection. Several data visualization techniques are used in cancer microarray data towards knowledge discovery and class labeled data analysis [15]. Among them, VizRank is a simple gene set ranking technique, which works through utilizing visual projections of class labeled data. Here, we employed Radviz (Radial Coordinate visualization)(Novakova and Stepankova, 2009 and Mramor et al., 2005) based gene identification with minimal gene numbers (three), to reduce computation cost, as well as to identify a subset of molecular criteria showing maximum efficacy which may potentially be implemented in routine diagnostics.

2. Materials and method

GSE30784 dataset was downloaded from Gene Expression Omnibus and used in this study, which consisted of 167 OSCC, 17 OED and 45 NOM samples. DE genes for each two class conditions were obtained using GEO2R (Barrett et al., 2013). The cut-off for gene selection was p value < 0.05 and log FC value ± 2. During 3 class disease classification, cut-off value was p value < 0.05 and F score more than 100.

Initially, all DE genes, both upregulated and downregulated gene sets were identified separately and then gene ontology (GO) analysis and pathway analysis for each gene set was performed using EnrichR (Chen et al., 2013) where common pathways as well as important biological process, cellular component and, molecular function were identified. In gene ontology (GO) analysis, when minimum of 5 genes were found to be present in any condition, was considered significant. When too many processes or functions were obtained, a threshold of combined score was considered and mentioned accordingly in the “Result and discussion” section. Pathway analysis was done using KEGG 2015 pathway. Common pathway and gene ontology analyses were performed with cut-off of combined score 25. The concept of combined score in EnrichR is to integrate both p value and z score with the formula $c = \log(p) \cdot z$ where c is the combined score, represented by p, p-value computed using the

Fisher exact test, and z the z-score computed by assessing the deviation from the expected rank. Since Enrichr provides all three options for sorting enriched terms, combined score of 25, and p value < 0.005 were only considered (Chen et al., 2013). Venn diagram was prepared using three different gene subsets, as well as six sub-sets of up- and down-regulated genes to identify common and exclusive genes in each process using InteractiVenn (Heberle et al., 2015). Utilization of this method aided understanding of the complexity of association of both upregulated and downregulated gene sets. GO analysis and pathway analysis for each gene set were also again performed using EnrichR (Chen et al., 2013).

During specific gene subset selection for optimal disease classification, efficiency of different supervised classifiers namely SVM, KNN and Naïve Bayes was assessed using best features obtained through weighted SVM feature ranking method. Efficiency of another feature ranking techniques namely ReliefF was compared with weighted SVM and plotted accordingly with the best classifier obtained in the previous step, SVM. For selection of best feature subset, manual sequential feature reduction was carried out and optimal classification efficiency was evaluated at 10 fold cross-validation. The gene set obtained for NOM and OSCC was cross-validated in GSE9844 data set, which comprised of 12 NOM and 26 tongue OSCC samples. These analyses were performed in Orange 2.7 (Demšar et al., 2004). Visualization based classification by Radviz with minimal gene numbers (three) was also performed. Plots have been provided in the supplementary figure. Biological functions of the genes obtained in this study have also been mined from Genecard (Safran et al., 2010) and presented in supplementary Tables 1, 2 and 3. The schematics of the entire process have been provided in Fig. 1.

3. Result and discussion

This study was performed towards utilization of gene ranking and visualization based precise gene set selection for comprehensive biological and bioinformatic knowledge fusion. The integrated approach of analysis was performed for cost minimization of specific gene signature selection from genomics information, which can be further validated by molecular pathology techniques or other relevant datasets.

For GO and pathway analysis, when DE genes were extracted, the result suggested that out of 54,675 genes, 829 genes were initially expressed in this study. The common pathway analysis suggested that cell communication was found to be affected in all conditions; while extra-cellular matrix (ECM) receptor interaction was commonly hampered in both NOM to OED and OSCC transition. The role of alteration of cell–cell adhesion and other intercellular communications, both in junction based and non-junctional modes, especially during epithelial mesenchymal transition during carcinogenesis is an established phenomenon, and thus was supported by the results (Kandouz, 2015 and Loewenstein and Kanno, 1966). Cytokine–cytokine receptor was also found to be affected in both NOM and OED to OSCC transition. A recent study validated the findings, since it confirmed that early cytokine–cytokine receptor induction is triggering factor of oral carcinogenesis (Liu et al., 2012). It is also evident from existing literature that deregulation in cell proliferation and cellular invasion, which hampered cellular differentiation, is associated with ECM dynamics which is found to be affected in fibrosis and cancer (Lu et al., 2011 and Sainio and Järveläinen, 2014). During biological activity analysis epidermis development (GO: 0008544) was found to be hampered in both NOM-DYS and DYS-CA process, while inflammatory response (GO: 0006954), taxis (GO: 0042330) and chemotaxis (GO: 0006935) were commonly hampered in NOM-CA and DYS-CA conditions. Eight GO terms extracellular matrix organization (GO: 0030198), extracellular structure organization (GO: 0043062), collagen metabolic process (GO: 0032963), multicellular organismal macromolecule metabolic process (GO: 0044259), multicellular organismal metabolic process (GO: 0044236), collagen catabolic process (GO: 0,030,574), multicellular organismal catabolic process (GO: 0044243) and extracellular matrix disassembly (GO: 0022617) was found to be altered in all processes. Recent studies suggested that intracellular collagen degradation is also associated with ECM turnover during malignancy due to altered μPARAP/Endo180 expression in mammary gland (Curino et al., 2005), which might be also the case here. A recent study also showed expression of

inflammation and ECM components in oral carcinogenesis and validated the notion obtained in this study (Tanis et al., 2014). The important affected biological process in OED associated oral carcinogenesis has been shown in Table 1, while Table 2 presented important affected cellular components in OED associated oral carcinogenesis obtained from GO analysis using of DE genes. Fig. 1 showed Venn diagram showing association of DE genes obtained in each two class conditions, NOM-OSCC, NOM-DYS and DYS-OSCC.

When common 28 cells common in all genes were subjected to GO analysis, it was found that in epithelial cell differentiation (GO: 0030855), epidermis development (GO: 0008544) and epithelium development (GO: 0060429), more or equal to 5 genes were involved. Extracellular matrix components were also involved and were in synergy with previous results (Banerjee and Chatterjee, 2015). Peptidase regulator activity (GO: 0061134), endopeptidase activity (GO: 0004175) and calcium ion binding (GO: 0005509) were the affected molecular function. Previously other network based studies showed activity of calcium binding proteins in oral carcinogenesis too (Nomura et al., 2007).

In all conditions, ECM related areas (extracellular region (GO: 0005576), extracellular space (GO: 0005615), extracellular vesicular exosome (GO: 0070062), extracellular matrix (GO: 0031012) and proteinaceous extracellular matrix (GO: 0005578)) were found to be affected. During NOM to OSCC transition, basement membrane (GO: 0005604) was also found to be involved. This concept supports a well-established fact that malignant potentiality is correlated with enzymatic degradation of basement membrane collagen (Liotta et al., 1980). Interestingly collagen related components were found to be affected in both NOM to DYS and OSCC transition, but not during DYS to OSCC conversion. It can be hence implied that collagen related alterations are prominent in early stages of carcinogenesis, while ECM related alterations are evident in all stages of. This result supports the preconceived result that in severe dysplasia and OSCC ECM is disintegrated, while collagen III and laminin play role in neo-angiogenesis in lung cancer (Fisseler-Eckhoff et al., 1990). The same mechanism is also likely to happen in oral carcinogenesis.

When the gene sets were segregated using Venn diagram with union by list specification and both upregulated and downregulated genes were utilized (depicted in Fig. 2 and Fig. 3), total 16 classes were identified and provided in Supplementary Table 4. For understanding the process of OED, role of the exclusive genes when evaluated, the gene ontology with combined score more than 5, it was found that negative regulation of proteolysis (GO: 0045861), negative regulation of protein processing (GO: 0010955), negative regulation of protein maturation (GO: 1903318), negative regulation of endopeptidase activity (GO: 0010951), negative regulation of peptidase activity (GO: 0010466), regulation of endopeptidase activity (GO: 0052548) and regulation of peptidase activity (GO: 0052547) was found to be hampered when biological activity was evaluated. Mostly endopeptidase activity is assaulted (Serine-type endopeptidase inhibitor activity (GO: 0004867) peptidase regulator activity (GO: 0061134), endopeptidase inhibitor activity (GO: 0004866), peptidase inhibitor activity (GO: 0030414), endopeptidase regulator activity (GO: 0061135) and enzyme inhibitor activity (GO: 0004857)) during molecular function assessment. In this regard a recent study suggested that expression of ADAMTS2 is important in craniofacial fibrous dysplasia (Zhou et al., 2014), while another study showed that ADAMTS2 is associated to regulation of procollagen amino-propeptide processing and affect collagen biosynthesis (Le Goff et al., 2006). Hence this information is in synergy with our result.

From the affected genes involved in NOM-DYS-UP and DYS-CA-UP, it was found that keratinization (GO: 0031424) was the most involved biological process, and mainly cell communication is affected in KEGG pathway. A recent study also revealed such associated alteration in oral carcinogenesis (Kandouz, 2015 and Banerjee et al., 2015).

Result shown in Fig. 4 depicted that NOM and DYS as well as NOM and OSCC can be clearly differentiated on the basis of principle components, but there were significant overlapping in DYS and OSCC conditions. Again in clinical theranostics, single genes would have better implication than principal components. So further single gene based analysis was initiated, but it could be understood that complete diagnostic segregation of DYS and OSCC is quite difficult.

When performances of the classifiers were tested, SVM showed maximum efficacy and thus was chosen for further analysis (Fig. 5a). When the role of two feature ranking method, ReliefF and weighted SVM were evaluated, weighted SVM showed better potential and thus was used for feature selection (Fig. 5b). Gene selection result showed that when NOM and OSCC were classified, best 23 features according to weighted SVM method could classify with 100% sensitivity and specificity. Further manual sequential feature reduction aided selection of six genes, which also showed similar efficacy and thus helped towards computational cost reduction. The gene sets with their corresponding weighted values in first bracket are PEG3 (0.037), UPK1A (0.018), LAMB1 (0.010), GREM1 (0.008), TYRP1 (0.007) and COMP (0.007). When the efficacy of the same gene set was validated using a different dataset, GSE9844 sensitivity was found to be 66.67%, specificity 88.46% and 81.67% accuracy, but further manual sequential feature reduction and elimination of LAMB1 and UPK1A resulted in significant betterment of the classification efficacy (sensitivity 91.67%, specificity 92.31% and accuracy 92.50%). The differences in the results are might be site specific variation in gene expression, since in that study only tongue cancers were considered (Ye et al., 2008). When Radviz score based projection with maximum three genes were performed, five gene sets were found to have score more than 99. The gene sets in the third bracket with their scores in the first bracket were [PEG3, MYBPC1, COL1A1] (99.26), [LOC100506098, MYBPC1, COL1A1] (99.19), [COL3A1, CHRDL1, PEG3] (99.14), [COL3A1, UPK1A, PEG3] (99.02), [LPIN1, COL1A1, PEG3] (99.00). Both results are far better than previous results, where PCA based weightage linked gene selection was performed in the same dataset to classify NOM and OSCC, while the best classification was found for gene set [MMP1, RUNX2, MTERFD2] with 98.80% sensitivity and 95.60% specificity (Kim et al., 2014).

During NOM and DYS classification, best 20 features could classify the conditions with 100% sensitivity and specificity too. Sequential feature reduction showed that two sets of 4 genes in each also provide the same results. The first gene set comprised of PRR9 (0.002), LMO7 (0.001), CAPN14 (0.001) and LOC344887 (0.001) while KRT10 (0.002), CRYM (0.002), LMO7 (0.001) and ATP6V0A4 (0.001) were present in the second list. Interestingly during Radviz scoring based classification with maximum three genes that were performed, ten gene sets were obtained with score of 100 and CDSN was common in all sets. The gene sets shown in the third brackets are [CDSN, CRYM, HYAL1], [CDSN, SLC8A1-AS1, ANKRD20A5P], [CDSN, F2RL2, FAM3D], [CDSN, PRR9, CAPN14], [CDSN, COL3A1, CEACAM1], [CDSN, FAM3D, LRRC15], [CDSN, FAM3D, HYAL1], [CDSN, FAM3D, ANKRD20A5P], [CDSN, ANKRD20A5P, CAPN14] and [CDSN, COL3A1, FAM3D]. CDSN, gene associated with epidermal barrier integrity was found to be most interesting in this set, which was also found to be present in all sets.

Diagnostic classification of DYS and OSCC using the gene expression was found to shown comparatively lesser efficiency than other two classes and more computation cost had to be exploited, since the number of genes in the subset was quite high. It was found that, the best 45 genes in weighted SVM could classify the lesions with 88.24% sensitivity, 99.40% specificity and 98.33% accuracy. Then a gene set of 32 genes was obtained which showed better efficacy by manual sequential feature selection (94.12% sensitivity, 99.40% specificity and 98.89% accuracy). Further feature reduction was also tried, but since specificity was found to be reduced nearly up to 1% (98.20%) and sensitivity of nearly 6% (88.24%) with 22 selected genes, in spite of the large number of genes, the former gene set was considered to be optimal. Although in previous studies the number of genes in the gene set was lesser, the sensitivity and specificity are higher in this study (Kim et al., 2014 and Chen et al., 2008). The result has been shown in Table 3. When the efficacy of Further then Radviz projection based classification was performed with maximum three number of genes, the gene set comprising BPIFC, PLEK2 and TNFAIP3 was found to be the only gene saving score greater than 94 (94.29).

Finally when three classes were tried to be segregated using linear SVM the result suggested that the best ranked 23 genes of weighted SVM feature ranking method could classify NOM, DYS and OSCC with 82.35% sensitivity and 99.53% sensitivity 96.53% accuracy. When manual sequential feature reduction was performed, it was found that a gene set containing 13, could classify the

conditions with 82.35% sensitivity and 100% specificity. The gene set with their corresponding weighted value in first bracket are LOX (0.823), 2NF519 (0.531), MTERF4 (0.498), F2RL2 (0.444), CLEC3B (0.418), AADAC (0.359), HSBP1 (0.345), NDNF (0.313), MMP1 (0.285), CRISP3 (0.269), ENAH (0.258), ATP2C1 (0.229) and PAQR8 (0.192). From the selected 4 gene sets, FAM3D were found to be common in NOM-OSCC and DYS-OSCC classes, COL3A1 were common in NOM-OSCC and NOM-DYS condition, which was found to be associated with cytokine activity and in collagen III expression respectively. Previously 5 gene sub-sets were identified in colorectal cancer stage specific classification (Berdiel-Acer et al., 2014), while this study is one of the detailed endeavor to identify gene sub-sets in oral cancer and their biological pathway and GO analysis.

4. Conclusion

It can be concluded that, knowledge discovery through integration of state-of-the-art data mining followed by meaningful biological interpretation of the result has been implemented in this study for understanding OED associated carcinogenesis. Utilization of both feature ranking and visualization technique aided identification of precise gene sets with minimum number of genes for optimal classification of two or three different conditions. Gene set selection was also performed towards minimization of arbitrary selection of gene sets in this respect. In turn, the selected gene sets in this study are expected to be used in routine clinical practice towards cost minimization of molecular pathology based oral lesion diagnostics..

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Comprehensive Selection of Reference Genes for Expression Studies in Meniscus Injury Using Quantitative Real-time PCR

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Abstract. The meniscus plays critical roles in the knee function. Meniscal tears can lead to knee osteoarthritis. Gene expression analysis may be a useful tool for understanding meniscus tears, and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) has become an effective method for such studies. However, this technique requires the use of suitable reference genes for data normalization. We evaluated the suitability of six reference genes (18S, ACTB, B2M, GAPDH, HPRT1 and TBP) using meniscus samples of (1) 19 patients with isolated meniscal tears, (2) 20 patients with meniscal tears and combined anterior cruciate ligament injury (ACL), and (3) 11 controls without meniscal tears. The stability of the candidate reference genes was determined using the NormFinder, geNorm, BestKeeper DataAssist and RefFinder software packages and comparative ΔCt method. Overall, HPRT1 was the best single reference gene. However, GenEx software demonstrated that two or more reference genes should be used for gene expression normalization, which was confirmed when we evaluated TGFβR1 expression using several reference gene combinations. HPRT1 + TBP was the most frequently identified pair from the analysis of samples of (1) meniscal tear samples of patients with a concomitant ACL tears, (2) all meniscal tears, and (3) all samples. HPRT1 + GAPDH was the most frequently identified pair from the analysis of samples of isolated meniscal tear samples and controls. In the analysis involving only controls, GAPDH + 18S was the most frequently identified pair. In the analysis of only isolated meniscal tear samples and in the analysis of meniscal tear samples of patients with concomitant ACL tears and controls, both HPRT1 + TBP and HPRT1 + GAPDH were identified as suitable pairs. If the gene expression study aims to compare non-injured meniscus, isolated meniscal tears and meniscal tears of patients with ACL tears as three independent groups, the trio of HPRT1 + TBP + GAPDH is the most suitable combination of reference genes.

Keywords: ACL, anterior cruciate ligament; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MRI, magnetic resonance imaging; TLDA, TaqMan Low-Density Array; AAV, adeno-associated virus; Crt, relative cycle threshold; Ct, cycle threshold; SD, standard deviation; CV, coefficient of variance; RQ, relative quantification; Acc.SD, accumulated standard deviation.

Keywords: Knee injury; Meniscal tears; Gene expression; Reverse-transcription quantitative polymerase chain reaction; Expression normalization; Reference genes.

1. Introduction

Menisci are important components in joint biomechanics with crucial roles in the knee joint: distributing joint forces, load bearing, and enhancing joint stability (Lee et al., 2014 and Kaleka et al., 2014). Lesion of this structure can cause pain, joint swelling, and osteoarthritis in the long term. Younger people are more likely to have acute lesions due to trauma, whereas older people are more likely to have lesions due to degeneration (Englund et al., 2009, Pauli et al., 2011 and Rai et al., 2013). Patients with traumatic meniscal tears commonly present an associated rupture of the anterior

cruciate ligament (ACL) (Poulsen and Johnson, 2011). Once present, meniscus tears are associated with an accelerated progression of cartilage degeneration in the knee compared with individuals with osteoarthritis but without tears (Biswal et al., 2002 and Hunter et al., 2006).

Recent studies have been performed to understand the gene expression alterations that may have a role in human meniscal tears. In a transcriptome analysis, several genes were identified that were differentially expressed with age and chondrosis in patients with meniscus tears (Rai et al., 2013a). Moreover, Brophy et al. investigated whether the expression of osteoarthritis markers (matrix components, cytokines, chemokines, aggrecanases, metalloproteinases, and transcription factor genes) are age- and sex-related in meniscal tears with and without a concomitant ACL tear (Brophy et al., 2012). The authors demonstrated that the meniscus in younger patients reacts with an intrinsic response and is more prone to inflammatory changes. Conversely, there were no differences in inflammatory cytokines or chemokines in the group of patients over forty years old (Brophy et al., 2012). If confirmed in larger studies, these markers may monitor local events at the surgical sites and detect osteoarthritis progression (Kambic, 2012).

Investigation of gene expression in human meniscal samples may help in improving the understanding of meniscal tears as well as osteoarthritis progression. Moreover, gene expression analysis will be important for guiding patient management and the development of new therapeutic options for these knee afflictions.

Although powerful techniques, including microarrays and high-throughput measurements, have been developed to detect gene expression levels, the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is commonly used in many laboratories (Wang et al., 2015). Moreover, because of its accuracy, sensitivity, and capacity for high-throughput analysis, RT-qPCR is currently considered to be the gold standard technique for evaluation of gene expression (Derveaux et al., 2010). RT-qPCR is one of the most commonly utilized approaches in functional genomics research, and its use in gene expression analysis may become more routine; furthermore, this technique is commonly used to validate data obtained by other methods (Kozera and Rapacz, 2013), including the data of transcriptomic analysis.

To obtain reliable data using RT-qPCR, a common method is to normalize the target gene expression using an endogenous reference gene. Ideally, reference genes should be stably expressed or at least vary only slightly in expression in all tissues or cells under the experiment conditions (Li et al., 2009); therefore, a validation experiment for the evaluation of reference gene expression stability for each target tissue and disease is recommended (Bustin and Mueller, 2005 and Hruz et al., 2011). Normalization with unstable internal controls may result in different values, leading to erroneous results (Yuzbasioglu et al., 2010). However, many authors do not critically evaluate their RT-qPCR experiments; therefore, the experiments are improperly designed and difficult to repeat because of insufficient data quality (Bustin, 2010). Consequently, the use of suitable reference genes with stable expression in the studied tissue (normal and/or injured) is essential for effective data normalization and the acquisition of accurate and meaningful biological data.

Suitability of reference genes has been evaluated in some human musculoskeletal diseases, such as shoulder instability (Leal et al., 2014), rotator cuff tears (Leal et al., 2015a), ACL tears (Leal et al., 2015b), osteoarthritic articular cartilage (hip and knee) (Pombo-Suarez et al., 2008), human lumbar vertebral endplate with modic changes (Zhou et al., 2014), and skeletal muscle with chronic degenerative changes (Yuzbasioglu et al., 2010). To our knowledge, no previous studies have described the best individual or set of reference genes for gene expression analysis in human meniscus samples. A previous study used GAPDH for gene expression normalization in meniscal tear samples of patients with and without a concomitant ACL tear (Brophy et al., 2012).

In this study, we assessed the suitability of six reference genes frequently reported in the literature (18S, ACTB, B2M, GAPDH, HPRT1 and TBP) using meniscus injured samples of patient with or without concomitant ACL tears as well as meniscus non-injured samples by analyzing gene stability with five software packages and comparative delta cycle threshold (ΔCt) method.

2. Materials and methods

2.1. Patients

Tissue samples were obtained from 39 patients with medial meniscal tears, including 19 samples of patients with isolated medial meniscal tears and 20 samples of patients with meniscus injury and a concomitant ACL injury. The following inclusion criteria were employed: age between 18 and 50 years old, clinical history compatible to meniscal injury (such as pain, swelling, stiffness, catching and locking), at least one specific physical examination test positive among McMurray (McMurray, 1949), Appley (Apley, 1947)and Steiman (Tria and Klein, 1992) tests that were used to diagnose meniscus injury (Speziali et al., 2015), magnetic resonance imaging (MRI) diagnosis of medial meniscus injury with abnormal signal extending to at least one articular surface involving the posterior horn and or the body of the medial meniscus (Crues et al., 1987), and arthroscopic confirmation of the medial meniscus lesion involving its posterior horn and or its body. The Lachman test (Torg et al., 1976), Anterior Drawer test (Marshall et al., 1975), and Pivot-Shift tests (Galway et al., 1972) were used to diagnose ACL injury (Astur et al., 2014a and Astur et al., 2014b). Coronal and sagital MRI view were used to identify meniscal and ACL lesions. All injuries were confirmed during arthroscopic procedure and reclassified if necessary.

The following exclusion criteria were also applied: medial meniscus lesions treated by suture (outside-in, inside-out or all-inside), medial meniscus stable lesions tested by probe palpation such as some longitudinal lesion < 1 cm, radial lesions < 5 mm, partial thickness lesions. The stable, unstable criteria was defined intra-operatively by the surgeon.

Additionally, 11 patients without any history of meniscal tears were included in this study as a control group. These patients were arthroscopically operated for other knee injuries, such as isolated ACL injury. All control patients were physically active. Table 1 displays the main clinical outcomes of the studied cases and controls.

This study was performed with the approval of the Ethics Committee of the Universidade Federal de São Paulo (UNIFESP), Brazil (CEP #51,436). Written informed consent with approval of the ethics committee was obtained from all patients prior to specimen collection.

2.2. Tissue samples

To collect tissue samples, patients were prepared in the standard fashion for arthroscopy meniscus surgery. A standard arthroscopic joint evaluation was carried out, confirming the meniscus injury or meniscus and ACL injuries diagnosis. During surgery, about 5 mm3 samples of the innermost part of the injured area of the posterior horn and the body of the medial meniscus were collected for gene expression analysis.

In the controls, a sample fragment of about 5 mm3 was resected from the innermost part of the healthy medial meniscus body by arthroscopy.

All tissue specimens were immediately immersed in Allprotect Tissue Reagent (Qiagen, USA) and stored at − 20 °C until RNA extraction.

2.3. RNA extraction

Total RNA was extracted from 10 to 20 mg of tissue sample using an AllPrep DNA/RNA/miRNA Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The mechanical lysis step was performed using the Tissue Lyser LT equipment (Qiagen, USA). RNA concentration and quality were immediately determined using a Nanodrop ND-1000 (Thermo Scientific, USA) and the integrity of the RNA was verified by gel electrophoresis on a 1% agarose gel. Aliquots of the total RNA were stored at − 80 °C until further use.

2.4. RT-qPCR

RT-qPCR gene expression quantifications were performed according to MIQE guidelines (Taylor and Mrkusich, 2014). Only RNA samples with the optical density (OD)260/280 > 1.8 were used, following the MIQE protocol.

First, cDNA was synthesized from 200 ng of RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) according to the manufacturer's protocol.

To detect the range of expression of the six candidate reference genes, reactions were performed with 75 ng of cDNA input using TaqMan Low-Density Array (TLDA) cards (Life Technologies, USA) and ViiA 7 Real-Time PCR System (Life Technologies, USA). Only inventoried TaqMan

Gene Expression Assays (Life Technologies, USA) were chosen for gene expression analysis. The final volume in each TLDA well is approximately 1 μl. All reactions were performed in triplicate.

To identify the best combination of reference genes, we also quantified the mRNA expression of target gene, TGFβR1, using the candidate reference genes for normalization. TGFβRI is considered one of the main receptor of TGFβ and has a key role in the canonical TGFβ signaling pathway (Moore-Smith and Pasche, 2016). Overexpression of TGFβ via adeno-associated virus (AAV) is capable of modulating the reparative activities of human meniscal cells, allowing for the healing of meniscal lesions (Cucchiarini et al., 2015). Therefore, TGFβR1 may also have a role in meniscal tears and healing. Other 9 target genes (TGFβ1, GDF5, COMP, TNC, TNXB, FN1, LOX, PLOD1 and PLOD2) were also evaluated to identify the best combination of reference genes (data not shown).

For each sample, the candidate reference and target genes were assayed on the same card to exclude technical variations. The 6 reference genes and target genes are summarized in Table 2.

The relative threshold method (Crt method) was applied, which is a robust method that sets a threshold for each curve individually based on the shape of the amplification curve, regardless of the height or variability of the curve during its early baseline fluorescence. The expression of TGFβR1 gene across the samples was calculated using the equation ΔCrt, in which [ΔCrt = target gene (TGFβR1) Crt− the mean of reference genes Crt]. A lower cycle threshold value (Crt) indicates higher gene expression.

2.5. Analysis of reference gene expression stability

We categorized the tissue samples into the following 7 groups: 1) isolated meniscal tear samples (N = 19); 2) meniscal tear samples of patients with a concomitant ACL tear (N = 20); 3) meniscal control samples (N = 11); 4) all injured meniscus (N = 39); 5) isolated meniscal tear samples and controls (N = 30); 6) meniscal tear samples of patients with a concomitant ACL tear and controls (N = 31); 7) all meniscus samples (N = 50). Typically, gene expression studies compare transcript levels between case (i.e., the injured tissue) and control samples, therefore we created the groups #5, #6 and #7. However, some researchers have been investigated a possible association between gene expression and clinical variables (Brophy et al., 2012), therefore we created the groups #1, #2 and #4. In addition, the group composed by only controls (group #3) was created since the understanding of gene expression regulation in non-injured ligaments is still necessary.

For comparisons of candidate reference gene stability we used the software programs NormFinder (http://123.233.119.36:80/rwt/119/http/P75YPLUNMSXC63DL/publicationsnormfinder.htm), geNorm (http://123.233.119.36:80/rwt/119/http/NWTXI35FNZYHK35FN34C6ZUF/~jvdesomp/genorm/), BestKeeper (http://123.233.119.36:80/rwt/119/http/P75YPLUHMWYGKLLSPWRX67DJM3SXC7DJN7YC63DF/bestkeeper.html) and DataAssist (http://123.233.119.36:80/rwt/119/http/P75YPLUMNFUGK7DFMNVG655MN7UXT3LUF3SX85B/us/en/home/technical-resources/software-downloads/dataassist-software.html) and the comparative ΔCt method (Silver et al., 2006). We also used the RefFinder software (http://123.233.119.36:80/rwt/119/http/P75YPLUMMWYX68DJMVYGG55N/referencegene.php) which integrates the results of geNorm, Normfinder, BestKeeper, and the comparative ΔCt method to compare and rank the tested candidate reference genes.

NormFinder accounts for both intra- and inter-group variations when evaluating the stability of each single reference gene (Andersen et al., 2004). The stability values and standard errors are calculated according to the transcription variation of the reference genes. Stably expressed genes, which have low variation in expression levels, present low stability values. NormFinder analysis also calculated the stability value for two reference genes.

geNorm calculates the expression stability value (M) for each gene based on the average pairwise expression ratio between a particular gene and all other reference genes. geNorm sequentially eliminates the gene that shows the highest variation relative to all the other genes based on paired expression values in all the studied samples. The most stably expressed gene yields the lowest M value, and then the two most stable reference genes are determined by stepwise exclusion of the least

stable gene (Vandesompele et al., 2002). Because of the elimination process, geNorm cannot identify a single suitable reference gene, and ends up by suggesting a pair of genes that shows high correlation and should be suitable for normalization of qPCR studies.

Bestkeeper was used to rank the 6 reference genes based on the standard deviation (SD) and coefficient of variance (CV) expressed as a percentage of the cycle threshold (Ct) level (Pfaffl et al., 2004). The more stable reference gene presents the lowest CV and SD. Bestkeeper also uses a statistical algorithm wherein the Pearson correlation coefficient for each candidate reference gene pair is calculated along with the probability of correlation significance of the pair.

DataAssist software provided a metric to measure reference gene stability based on the geNorm algorithm. In contrast to the other programs, DataAssist uses the relative quantification (RQ) to calculate the stability value of individual candidate reference genes. The lower score represents the more stable the control.

The comparative ΔCt method is based on the comparing relative expression of pairs of possible reference genes within each sample. The stability of the candidate housekeeping genes is ranked according to reproducibility of the gene expression differences among studied samples.

Lastly, RefFinder assigns an appropriate weight to an individual gene and calculated the geometric mean of their weights for the overall final ranking based on the rankings from geNorm, Normfinder, BestKeeper, and the comparative ΔCt.

GenEx software (http://123.233.119.36:80/rwt/119/http/M7TX63LZF3UXK5UFFWZYKZLPPSVXMZ5BPSVX85SPNFYGM5D/) was used to determine the optimal number of reference genes by calculating the accumulated standard deviation (Acc.SD). If larger number of reference genes is used, random variation among the genes' expression partially cancel reducing the SD. A minimum in the Acc.SD plot indicate the number of reference genes that give the lowest SD.

2.6. Statistical analysis

To compare TGFβR1 expression between the groups, we first verified the distribution of the data using the Shapiro–Wilk normality test for the determination of the appropriate tests for the subsequent statistical comparisons. TGFβR1 expression (ΔCrt) was not normally distributed. Therefore, the Mann–Whitney test was performed to compare TGFβR1 expression between the studied groups. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Reference gene expression levels

The distribution of Crt values for each of the 6 candidate reference genes is shown in Fig. 1. These genes displayed a wide range of expression levels. 18S presented the highest expression level among the candidate reference genes (mean Crt value ± SD: 12.52 ± 2.34). In contrast, TPB (31.16 ± 1.61) and HPRT1 (31.85 ± 1.79) presented the lowest expression level in meniscal samples.

3.2. Reference gene expression stability

Table S1 displays the stability value ranking of single candidate reference genes as determined by the different software packages and comparative ΔCt method. In our analysis, all the reference genes for all analysis groups presented M values less than the geNorm threshold of 1.5, which is recognized as stable (Table S1). However, 18S, ACTB, and B2M presented high SD (± x-fold) of Crt in the analysis involving all samples according to BestKeeper software [SD (± x-fold) = 3.21, 3.64, and 3.41, respectively), whereby any studied gene with SD (± x-fold) > 3 can be considered inconsistent. Furthermore, 18S, ACTB, and B2M presented high SD (± x-fold) in the analysis of (1) all meniscal tear samples, (2) isolated meniscal tears, and (3) isolated meniscal tears and controls. Moreover, ACTB and B2M presented high SD (± x-fold) in the analysis of (1) meniscal tear samples of patients with concomitant ACL tears and (2) meniscal tear samples of patients with concomitant ACL tears and controls. ACTB demonstrated high SD (± x-fold) in the analysis involving only controls.

Although none of the software packages and comparative ΔCt method suggested the same rank of reference genes in the studied sample groups, the methods applied did generate similar rankings of reference gene stability for each analysis group (Table S1).

Table 3 shows the best suitable reference gene by the different methods applied. In the present study, HPRT1 was the most suitable reference gene for the meniscus samples. As previously described, gene expression studies typically compare transcript levels between injured and non-injured tissue samples. When the isolated meniscus tear samples and controls were evaluated together, HPRT1 and GAPDH were the most suitable reference genes. When considering the meniscal tear samples of patients with a concomitant ACL tear and controls, TBP followed by GAPDH was the most stable gene. When considering all injured meniscal tear samples and controls (all samples), HPRT1 followed by TBP was also the most stable gene (Table 3; Table S1).

When we individually evaluated each group of meniscus samples, we observed that HPRT1 followed by TBP was the most stable gene for the isolated meniscal tear samples as well as for meniscal tear samples of patients with a concomitant ACL tear. In the control group, GAPDH and 18S were the most stable genes. HPRT1 followed by TBP was the most suitable gene in the analysis involving all meniscal tear samples (Table 3; Table S1).

3.3. Analysis of the best combinations of reference genes

Table 4 displays the best combinations of reference genes, as suggested by the software packages, comparative ΔCt method, and by visual inspection of all the ranks generated by these analyses. Overall, HPRT1 + TBP and HPRT1 + GAPDH pairs of genes were the most frequently identified pairs. HPRT1 + TBP was the most frequently identified pair from the analysis of samples from (1) meniscal tear samples of patients with a concomitant ACL tear, (2) all meniscal tears, and (3) all samples. HPRT1 + GAPDH was the most frequently identified pair from the analysis of samples from isolated meniscal tear samples and controls. In the analysis involving only controls, GAPDH + 18S was the most frequently identified pair. In the analysis of only isolated meniscal tear samples and in the analysis of meniscal tear samples of patients with concomitant ACL tears and controls, no more than two methods agreed in the definition of the best pair (Table 4).

The NormFinder, GeNorm, DataAssist, and BestKeeper software packages only indicated up to 2 genes as the best combination of reference genes. By visual inspection of all the ranks generated, software used, and comparative ΔCt method, we observed that HPRT1 + TBP + GAPDH was more frequently the best trio of reference genes. HPRT1 + TBP + 18S was the best trio only in the analysis of meniscal tear samples from patients with concomitant ACL tears. Additionally, GAPDH + TBP + 18S was the best trio in the analysis involving only control samples.

We used the GenEx software package to determine the appropriate number of reference genes for a reliable normalization. In this analysis, the optimal number of reference genes is indicated by the lowest SD. In all analyses, the Acc.SD of two reference genes did not differ by > 0.1 from the observed metric when using more than two genes (Fig. 2). However, one reference gene is not suitable for gene expression normalization in the analysis involving (1) isolated meniscal tears and controls or (2) meniscal tears of patients with concomitant ACL tears and controls. In these groups of samples, the Acc.SD of one reference gene was > 0.1 from the observed metric when using two or more genes (Fig. 2). Moreover, in the analysis involving all meniscal samples, the Acc.SD of one reference gene was > 0.1 from the observed metric when using four or more genes (Fig. 2). In the analysis of meniscal tears of patients with concomitant ACL tears, the Acc.SD of one reference gene was also > 0.1 from that observed when using more than three genes, which presented the lowest Acc. SD.

3.4. Effects of reference gene choice

To evaluate the effect of appropriate reference gene selection, an expression analysis was performed by comparing data from 1) meniscal tears from patients with and without a concomitant ACL tear, 2) isolated meniscal tear samples and controls, 3) meniscal tear samples of patients with a concomitant ACL tear and controls, and 4) injured meniscus samples and controls. This analysis was performed using TGFβR1 as the target gene in all the analyses. For reference genes, we used the most frequently identified pairs described above (HPRT1 + TBP, GAPDH + 18S, HPRT1 + GAPDH, and GAPDH + TBP). We also performed collagen genes expression analysis using three reference genes (HPRT1 + TBP + GAPDH, HPRT1 + TBP + 18S, and GAPDH + TBP + 18S), four reference genes (HPRT1 + TBP + GAPDH + 18S), and all studied reference genes. TGFβR1 was also normalized by

only 18S, ACTB, GAPDH, and HPRT1, as commonly described in the literature involving joint lesions. B2M + 18S was used as an example of a not suitable reference gene pair for gene expression normalization in meniscus samples.

Although the normalized expression quantities differed between the various combinations of reference genes, the distributions of the target gene expression in the studied samples were similar (Fig. 3).

Table 5 shows TGFβR1 expression using the different reference gene combinations for data normalization. TGFβR1 expression was significantly increased in the isolated meniscal tear samples compared with the controls when using several reference gene combinations ($p < 0.05$), except for HPRT1 ($p = 0.121$), HPRT1 + TBP ($p = 0.061$), and B2M + 18 ($p = 0.077$).

Alternatively, TGFβR1 expression was significantly increased in the meniscal tear samples from patients with concomitant ACL tears compared with the controls only when using GAPDH ($p = 0.0212$) as a reference gene. Moreover, all injured meniscal samples presented increased TGFβR1 expression compared with controls only when the target expression was normalized by GAPDH ($p = 0.008$) or GAPDH + 18S ($p = 0.043$).

When we compared isolated meniscus tear samples and meniscal samples from patients with ACL tears, TGFβR1 was significantly different between groups when its expression was normalized by B2M + 18S ($p = 0.049$).

4. Discussion

Comparing gene expression in different samples may generate misleading information related to distinctive amounts of material, RNA extraction and efficiency of reverse transcription. To overcome those risk potentials and be accurate, RT-qPCR relies on normalization to an internal control, often referred to as a housekeeping gene. Housekeeping genes are constitutive genes which are transcribed at a relatively constant level. Its products are vital to the basic functions of a cell. It is generally assumed that their expression is minimally affected by experimental conditions. Related to these characteristics, some candidates housekeeping genes are used on various studies to have its stability ranked according to the reproducibility of differences in gene expression among the studied samples. This stability analysis is helps to select the most appropriate candidate reference gene (Leal et al., 2014; 2015a; 2015b).

Reference genes have been described for RT-qPCR studies in several diseases and tissues (Yuzbasioglu et al., 2010, Pombo-Suarez et al., 2008, Zhou et al., 2014, Lyng et al., 2008, Rubie et al., 2005, Fu et al., 2010, Wang et al., 2012 and Wisnieski et al., 2013), and our group recently identified the most stable reference genes in the glenohumeral capsule of patients with and without shoulder instability (Leal et al., 2014), in tendon of patients with and without rotator cuff tears (Leal et al., 2015a) and in ACL of patients with or without ACL tears (Leal et al., 2015b). To the best of our knowledge, no prior study has aimed to identify suitable reference genes for gene expression analyses by quantitative approaches in the human meniscus.

In the present study, we used five software packages (NormFinder, geNorm, BestKeeper, DataAssist, and RefFinder) and the comparative ΔCt method to evaluate the stability of reference gene expression. As each analysis uses distinct algorithms, different results can be expected. Therefore, it is important to use more than one software package/method to identify the most suitable reference genes among a set of candidates. Although the distinct analysis differed in the rankings of reference gene stability as well as in the identity of the most suitable pair, at least two programs produced results that agreed for almost all the analyses. Our results demonstrate that the use of 5 statistical tools and comparative ΔCt method aids in the identification of the best reference genes.

Surprisingly, Normfinder, geNorm, and BestKeeper from RefFinder did not lead to the same output as we got from our NormFinder, geNorm, and Bestkeeper interface (data not shown), which is probably due to different versions of the algorithm. This lack of agreement was previously reported in literature (Llanos et al., 2016).

All the reference genes in this study were recognized as stable by geNorm analysis under the different experimental conditions tested. However, 18S, ACTB, and B2M were not suitable by

BestKeeper software in most of the groups of analyses. According to BestKeeper analysis, these reference genes should not be used in studies involving isolated meniscal tears, meniscal tears of patients with and without concomitant ACL injury, as well as in a study involving all types of meniscal samples investigated here.

In the different groups of analyses, HPRT1 appeared to be the most suitable gene overall. GAPDH was also previously used as a reference gene in a gene expression study from human meniscal tears with and without concomitant ACL injury (Brophy et al., 2012); however, this gene was the most stable only in some analyses with meniscal control samples and with isolated meniscal tear samples and controls.

It is increasingly clear that in most situations, a single reference gene is not sufficiently stable (de Jonge et al., 2007). Here, we observed that the use of one reference gene is not appropriate, mainly for the analyses involving meniscal tear samples and controls.

In our study, a pair of reference genes seems to be suitable for gene expression normalization in the studied groups. When a larger number of reference genes is used, the SD of the normalization factor (mean of reference gene expression) is reduced, and the random variation among the expression of the tested genes is partially canceled. As the inclusion of additional reference genes increases the time and money required for the analysis, it is important to consider the degree of improvement and overall noise contributed by reference genes when deciding how many reference genes are required. Considering the reproducibility of real-time PCR equipment, we believe that the use of several reference genes does not significantly improve the data quality. However, it is important to highlight that we observed the use of one, two, three, or more reference genes may lead to differences in the statistical analysis result of some group comparisons.

Although different combinations of reference genes were determined as being the most suitable for the various analysis, HPRT1 + TBP and HPRT1 + GAPDH were the most frequently identified pair, and HPRT1 + TBP + GAPDH was the most frequently identified trio. The selection of the appropriate pair or trio should consider the group of meniscus samples that will be investigated. For example, HPRT1 + TBP + 18S was the best trio only in the analysis of meniscal tear samples of patients with concomitant ACL tears, and GAPDH + TBP + 18S was the best trio in the analysis involving only control samples, which is in agreement with the observation that 18S may be stable in these groups according to BestKeeper software.

To identify the best combination of reference genes, we evaluated TGFβR1 expression in samples from meniscus tissue of the cases and the controls. Statistical comparison revealed that TGFβR1 expression differed between the isolated meniscal tear samples and the controls when using several reference gene combinations ($p < 0.05$), except for HPRT1, HPRT1 + TBP, and B2M + 18. This finding reinforces the use of one reference gene (even the most stable) is not appropriate for gene expression normalization in this group of samples. Moreover, HPRT1 + TBP and B2M + 18 were not the best pair in this group of samples. Only geNorm suggested that HPRT1 + TBP were the most stable genes in the analysis involving isolated meniscal tear samples and controls.

HPRT1 + TBP was the best pair of reference genes in the analysis involving all injured meniscus samples and controls, but not when meniscus tear samples from patients with or without ACL injury were grouped separately with controls. Consequently, if a gene expression study aims to compare non-injured menisci, isolated meniscal tears and meniscal tears of patients with ACL tears as three independent groups of analysis, HPRT1 + TBP + GAPDH seems to be the most suitable combination of reference genes. This trio of reference genes was the most suitable for the different comparisons involving injured and non-injured meniscus samples, as well as in the analysis involving injured meniscus samples of patients with and without ACL tears.

When meniscal tear samples of patients with concomitant ACL tears were compared with the controls, no significant difference was detected, except when TGFβR1 expression was normalized only by GAPDH. The use of GAPDH as well as GAPDH + 18S for normalization also lead to the observation that TGFβR1 expression differed between meniscal tear samples and controls. As already discussed, these analyses highlight the necessity of the selection of suitable reference genes based on the studied meniscus samples.

During this study, we also evaluated the use of different reference gene combinations in the expression of other nine extracellular matrix genes (data not show). No significance difference was detected between the studied groups using the different reference gene combinations.

Our study presented some limitations. First, we only included a limited number of candidate reference genes, and it is likely that some other genes may also be used as internal references for gene expression studies in meniscus samples from patients with or without history of a meniscal tear. Second, the number of samples available for the Mann–Whitney test was reduced, especially in the control group. However, to the best of our knowledge, no prior study evaluated RNA expression in human non-injured meniscus samples. Nevertheless, it is important to highlight that our results may be relevant to the study of meniscal tears, as well as to the study of normal menisci.

4. Discussion

The use of suitable reference genes for a reliable gene expression evaluation using RT-qPCR should consider the type of meniscus samples investigated (injured or non-injured). HPRT1 was the most suitable reference gene. However, the use of only one reference gene does not seem suitable for gene expression normalization in meniscal tear studies. HPRT1 + TBP and HPRT1 + TBP + GAPDH were the best combination of reference genes for the analysis of involving meniscus samples. However, if the gene expression study aims to compare non-injured menisci, isolated meniscal tears and meniscal tears from patients with ACL tears as three independent groups, HPRT1 + TBP + GAPDH is the most suitable combination of reference genes. The results of this work may benefit future studies of the meniscus that require more accurate gene expression quantification.

The following is the supplementary data related to this article.

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* Corresponding Author: Prof. Name, PhD / M.D.. E-mail: abc@abc.com

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$$c^2 = a^2 + b^2. \quad (1)$$

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