VALIDATION OF TISSUE MICROARRAY FOR IMMUNOHISTOCHEMICAL ANALYSIS OF ORAL SQUAMOUS CELL CARCINOMA BY USING VIRTUAL CORES

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ABSTRACT

Original Article

Background: There is significant amount of research done on Oral Squamous cell carcinoma (OSCC). One research technique is immunohistochemical (IHC) analysis using whole sections. With little availability of OSCC tissues high throughput analysis such as Tissue Microarray (TMA) are capable of efficient analysis of small samples. However, the results become questionable if the tumor exhibits high degree of heterogeneity as TMA cores might not accurately represent the whole section. **Aim:** The aim of this study is to determine the optimal number of TMA cores required to provide an accurate representation of the whole section with IHC analysis in OSCC. **Materials and Methods:** Twenty tissue samples stained with anti-p53 antibody were scanned at 40x magnification. Three to six virtual cores of size 0.6 mm, 1.0 mm and 1.5 mm were drawn on the scanned slides. H-scores were obtained for both whole sections and cores using NuclearQuant (3DHistech, Budapest, Hungary) software after eliminating non-tumour cells and artifacts manually. The correspondence between the cores and whole sections were calculated using intra-class correlation and one sample t-test. **Results:** Good correlation was obtained with just a single core of 0.6mm (0.826). Subsequent increase in core number and size resulted in improved correlation coefficient and smaller confidence interval. **Conclusion:** Three TMA cores of 0.6 mm would be the most optimal, as not only was there very strong correlation with the whole tissue section, the extra core will also be able to act as confirmation if the results of the first 2 cores are in doubt.

Keywords: Tissue microarray, immunohisto-chemical analysis, oral squamous cell carcinoma, p53 tumor marker, oral cancer.

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INTRODUCTION

Oral cancer (OC) is the 6th most common cancers in the world (1). In Malaysia, the National Cancer Registry Report 2007 reported that OC ranked 16th and 17th most common cancer among females and males in Peninsular Malaysia. The Indian population is most susceptible to OC and it ranks as the 4th most common cancer among Indian females and 8th most common among Indian males (2). Oral squamous cell carcinoma (OSCC) accounts for over 90% of OC cases.

The carcinogenesis of OSSC is still not well understood and over the years, a lot of research has been carried out. One of the most widely used research method is immunohistochemical (IHC) analysis of the cancer cells. Immunohistochemistry refers to the process of detecting antigens in cells by using antibodies that bind specifically to the intended antigen (3). This principle has been known since the 1930s but it was not until 1942 that the first IHC study was reported. Coons *et al* (1942) used Fluorescein Isothiocyanate (FITC) antibodies to identify *Pneumococcal* antigens in infected tissue (4). Since then, improvements and refinements have been made in this field and it is now used as a routine diagnostic tool in most laboratories and also widely used in research.

P53 is a tumor suppressor protein, which in humans, is coded by the TP53gene (5). In healthy humans, the p53 protein is continually produced and degraded in the cell. However, mutant p53 proteins which often manifests in cancer are unregulated and not degraded as normal p53 proteins and are thus able to accumulate at very high

concentrations (6). The p53 protein marker binds to the expressed protein in the amount corresponding to the extent of expression.

The gold standard for tumor research is whole tissue staining. By this method, the whole specimen is stained and analyzed. The result is a totally accurate representation of the tumor that is in question. This is acceptable for most tumors that have large areas to be experimented on. However, in the case of small tumors such as OSCC, there is limited tissue which can be used for research, hence the need for tissue conserving methods that optimize the usage of available oral tissues. Tissue Microarray (TMA) for sample preparation is one such technique.

The concept of TMA was first described by Battifora in 1986 as a 'sausage' method for laboratory tests of large samples (7). However, the current shape of TMA was defined by Kononen *et al* in 1998 when he described a single paraffin block containing around 1000 different yet uniformly-sized tissue samples. These blocks then produced many slides for experimentation (8).

The advantage of TMA is that multiple tissue samples can be stained and analyzed at the same time. This reduces laboratory errors as well as observer bias as the process is done at the same time to all the tissue specimens (9). Furthermore, the efficiency is also maximized in that a few slides can generate the amount of data that will conventionally require hundreds of slides to obtain. Most of the conventional stain and reagents can be used on TMA cores as with the conventional whole sections (10). As fewer slides are required to produce the data; reagents, tissue and time are dramatically reduced. This technique has successfully been used to develop institution-specific staining profiles for various antibodies as quality assurance analysis (11).

However, the number of cores to be used is controversial especially in tumors that exhibit a high degree of heterogeneity such as OSCC. It is of concern that TMA cores will not fully represent the whole section (12). Many studies have tried to address this issue in regard to other cancers that exhibit high heterogeneity. Camp *et al* (2000) suggested two 0.6 mm cores were sufficient in representing the whole section when studying breast carcinoma (13). However, Torhorst *et al* (2001) and Zhang *et al* (2003) found that a single core of 0.6 mm was sufficient (14, 15). Hoos *et al* (2001) studied various fibroblastic tumors and recommended three 0.6 mm cores to achieve a 96-98% concordance (16). Similar results were observed by Fernebro *et al* (2002) when studying rectal cancer (17) and by Proverbs-Singh *et al* (2001) in prostate cancer (18). Nocito *et al* (2001) observed that four 0.6 mm cores were extremely concordant with the whole section in bladder cancer (19). Griffin et al (2003) used p53 immunostaining on squamous cell carcinoma of the larynx to validated TMA (20). With regards to OSCC, Monteiro *et al* (2010) conducted a study validating this technique and found that two 1.5 mm cores adequately represented the whole sections (21). However, no further studies were found relating to this topic. Therefore in this study we aim to determine the optimal number of TMA cores required to provide an accurate representation of whole sections with IHC analysis of OSCC by using virtual TMA cores.

MATERIALS AND METHOD

Tissue samples:

Twenty samples of OSCC tissues which were stained with anti-p53 antibody were selected for this study. These tissues were obtained from a previous study (22). These samples were scanned using the Pannoramic Desk Scanner (3DHistech, Budapest, Hungary) with magnification of 40x (Figure 1).

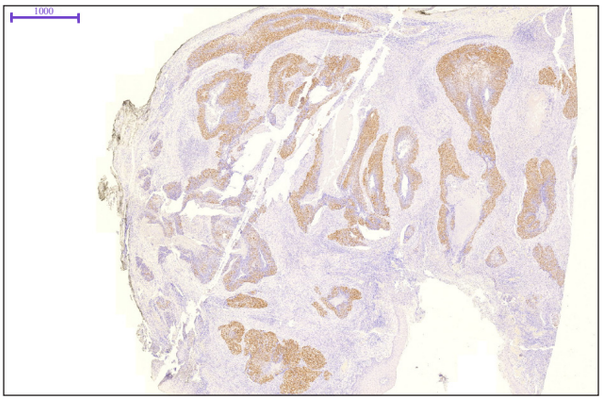

Figure 1: Photomicrograph of OSCC tissue stained with anti-p53 antibody and scanned using Pannoramic Desk Scanner (3DHistech, Budapest, Hungary) (Magnification 4x)

Tissue Microarray:

On each of the tissue samples, virtual cores of 3 sizes were drawn. The sizes are 0.6mm, 1.0mm and 1.5mm in diameter. The number of cores ranged from 3 to 6 cores depending on the size of the tissue sample (Figure 2). Before placing the cores, the area of interest were pre-selected to include tumor areas and the same was confirmed by an Oral Pathologist (AR).

The tumor cells were scored according to their intensity of staining as 0 (negative), +1 (weakly positive), +2 (moderately positive) and +3 (strong positive) by using the NuclearQuant (3DHistech, Budapest, Hungary) software (Figure 3). This software isolated the nucleus of each cell and places them in a gallery (Figure 3) for easy scoring which was followed by manual scoring of each cell to obtain the final score. During the process of manual scoring non-tumor cells such as the inflammatory cells, fibroblasts and artifacts were identified and deleted from the gallery.

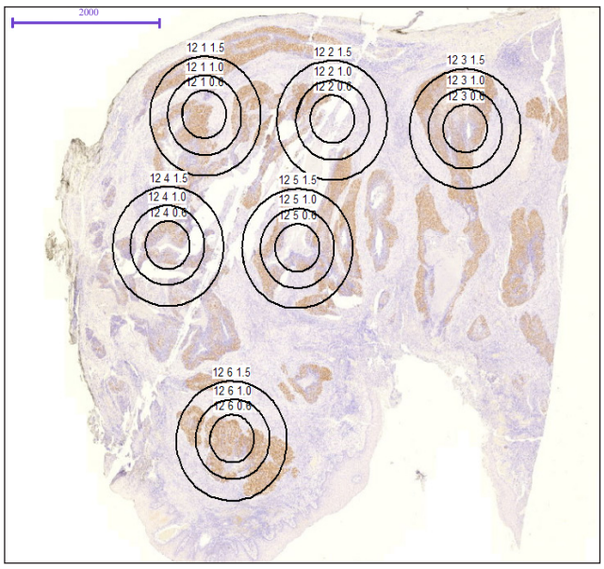

Figure 2: Photomicrograph showing 6 cores of 3 different sizes (0.6 mm, 1.0 mm and 1.5 mm in diameter) drawn on the OSCC whole tissue sample (Magnification 4x)

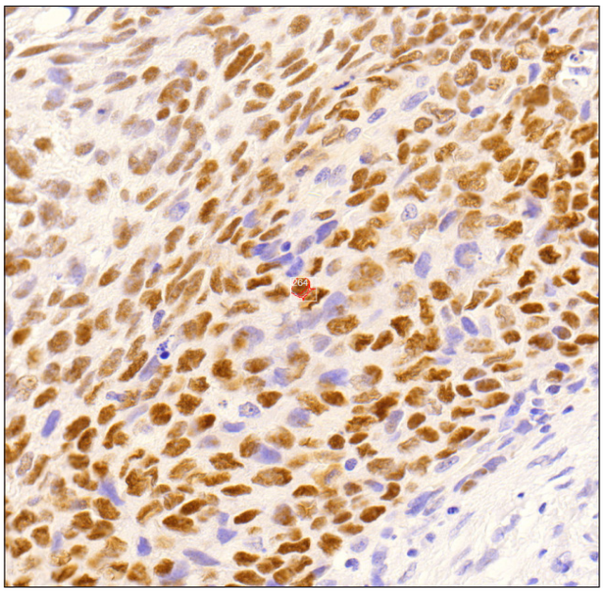

Figure 3: Photomicrograph showing isolation of nuclei and initial scoring with NuclearQuant (3DHistech, Budapest, Hungary) according to staining intensity

The analysis of the whole tissue section was performed and the area of interest, which is the tumor areas were marked out and the procedure was repeated (Figure 4). The NuclearQuant (3DHistech, Budapest, Hungary) software was first used to isolate the cells' nucleus followed by the manual scoring and elimination of the non-tumor cells and artifacts. The H-score, which is a semi-quantitative method of assessing the extent of nuclear immunoreactivity, for each core and the whole tissue section were calculated using the NuclearQuant (3DHistech, Budapest, Hungary) software. The H-score was calculated using the following formula:

H-Score = (% at 0) * 0 + (% at 1+) * 1 + (% at 2+) * 2 + (% at 3+) * 3.

STATISTICAL ANALYSIS

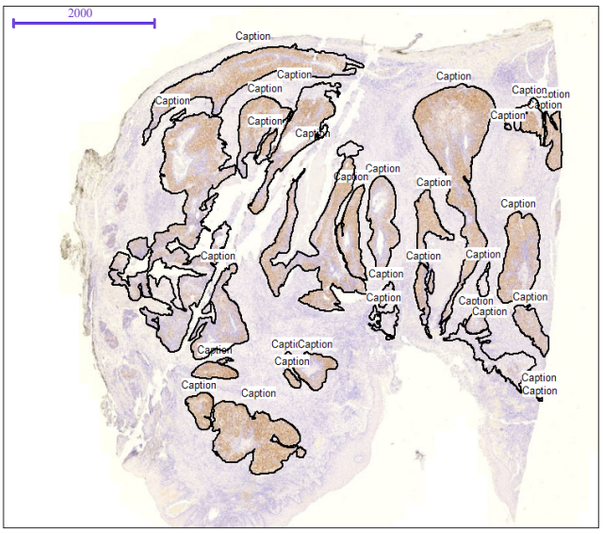

Figure 4: Photomicrograph of whole tissue section of OSCC showing the tumor areas marked (Magnification 4x)

The results were analyzed using SPSS version 20. The H-scores as well as the number of positive and negatively stained cells for each core and the whole tissue section were recorded. The mean H-scores for two, three, four, five, and six cores for all three sizes (0.6 mm, 1.0 mm, and 1.5 mm) were calculated. Using those values, the concordance between the TMA core samples and the whole tissue sections were calculated using one sample t-test and intra-class correlation with the H-score as the measurement unit.

RESULTS

Out of the 20 tissue samples that were scanned in this study, 13 samples were large enough to contain 6 TMA cores. The others were excluded from the study for uniformity. The results are as shown. Supplementary Tables 1, 2, and 3 are provided showing the mean H-score obtained from the TMA cores of all 3 sizes and whole section.

It was observed from the intra-class correlation statistical analysis (Table 1), that a single core of 0.6mm diameter sufficiently represents the whole tissue section with a coefficient of 0.828. However, it is noted that with

the increase in the number of cores used, the correlation coefficient improved. When two, three, four, five and six cores of 0.6mm diameter were used, correlation coefficients of 0.898, 0.939, 0.959, 0.958, and 0.957 were obtained respectively. Similar patterns of results were seen when cores of sizes 1.0mm and 1.5mm were used. One core of 1.5mm diameter showed a correlation coefficient of 0.860 whereas six cores of 1.5mm diameter gave a higher correlation of 0.963.

The increase in size of the cores also gives a stronger association. With one core of 0.6mm diameter, a correlation of 0.828 is attained as opposed to one core of 1.5mm diameter which gives a correlation of 0.860 between the TMA core and the whole section.

Table 1: The intraclass correlation between the whole tissue sections (WS) and TMA cores of different sizes (0.6 mm, 1.0 mm and 1.5 mm)

	Whole Sections Vs. 0.6 mm cores					
	1 core (n=13)	**2 cores (n=13)**	**3 cores (n=13)**	**4 cores (n=13)**	**5 cores (n=13)**	**6 cores (n=13)**
Correlation Coefficient	0.828	0.898	0.939	0.959	0.958	0.957
p-value	0.002	0.000	0.000	0.000	0.000	0.000
95% Confidence Interval	0.435-0.947	0.666-0.969	0.799-0.981	0.864-0.987	0.863-0.987	0.857-0.987
	Whole Sections Vs. 1.0 mm cores					
Correlation Coefficient	0.818	0.891	0.926	0.866	0.955	0.955
p-value	0.003	0.000	0.000	0.001	0.000	0.000
95% Confidence Interval	0.405-0.945	0.644-0.967	0.759-0.978	0.516-0.959	0.853-0.986	0.853-0.986
	Whole Sections Vs. 1.5 mm cores					
Correlation Coefficient	0.860	0.901	0.939	0.952	0.959	0.963
p-value	0.001	0.000	0.000	0.000	0.000	0.000
095% Confidence Interval	0.540-0.957	0.674-0.970	0.800-0.981	0.841-0.985	0.867-0.988	0.878-0.989

Furthermore, as the number of the TMA cores increases, the range of the 95% confidence interval (CI) becomes smaller. When only one core of 0.6mm diameter is used, the 95% CI ranges from 0.51 whereas, with 6 cores, a 95% CI from 0.13 is achieved. Similar patterns were also observed when cores of size 1.0mm and 1.5mm diameters were used. The 95% CI becomes smaller with the increase in the size of the core from 0.6mm to 1.5mm diameters.

One sample t-test (Table 2), also demonstrates good concordance between a single core of the TMA and whole sections. P-value of >0.05 were obtained with just one core of different sizes (0.6mm, 1.0 mm and 1.5 mm) signifying that there is no significant difference between one core and the whole section.

Table 2: *p*-values of one sample t-test between the whole tissue sections (WS) and TMA cores of different sizes (0.6 mm, 1.0 mm and 1.5 mm)

	1 core	**2 cores**	**3 cores**	**4 cores**	**5 cores**	**6 cores**
Whole Sections Vs. 0.6 mm cores	0.825	0.787	0.891	0.895	0.672	0.736
Whole Sections Vs. 1.0 mm cores	0.758	0.789	0.817	0.593	0.895	0.823
Whole Sections Vs. 1.5 mm cores	0.784	0.840	0.972	0.768	0.928	0.995

Supplementary Table 1: The mean H-scores for 0.6 mm TMA cores and the whole sections for 20 samples

Slide	Size	Sample No.	Six Cores	Five Cores	Four Cores	Three Cores	Two Cores	One Core	Whole Section
tma a1-2	0.6mm	1	161.535	160.842	168.9801	160.1311	175.4477	194.8953	191.0037
tma a1-2	0.6mm	2	78.2946	77.6572	82.5796	86.4885	95.847	116.16	32.528
tma a1-5	0.6mm	3				8.6097	6.0786	3.4298	176.0478
tma a1-3	0.6mm	4				40.215	29.934	29.5181	116.1594
tma a1-4	0.6mm	5	97.9584	79.2659	94.7871	123.1935	180.0722	207.5758	50.39
tma a1-5	0.6mm	6			192.4487	180.4001	178.8325	162.4595	21.361
tma a1-7	0.6mm	7	75.7081	72.5179	66.2334	64.4829	61.9088	61.2216	77.0994
tma a1-8	0.6mm	8	149.6573	149.732	148.8029	148.3364	158.8472	215.7895	104.4944
tma a2-7	0.6mm	9			211.8551	209.8587	201.9382	226.1137	185.7361
tma a2-9	0.6mm	10				18.6495	24.4217	29.1332	25.1934
tma a1-3	0.6mm	11				40.2149	9.934	29.5181	17.9218
tma a8-3	0.6mm	12	207.4795	206.7077	212.3231	214.5001	199.5934	230.5019	191.2527
tma a8-3	0.6mm	13	219.112	221.8273	222.3188	223.5856	201.4623	152.2222	143.0293
tma a8-3	0.6mm	14	256.4484	258.2212	250.1004	241.4984	237.0382	269.2063	214.2186
tma a8-5	0.6mm	15	20.7531	23.6778	20.1593	13.1311	14.216	20.4276	13.4525
tma a3-3	0.6mm	16	81.2919	81.0418	91.5753	94.0831	111.4679	146.384	73.0539
tma a3-3	0.6mm	17	71.8919	70.2771	77.8054	91.0898	96.8234	124.1176	44.8392
tma a4-4	0.6mm	18	166.6509	158.4107	139.1714	142.9095	138.2181	88.853	115.9934
tma a4-4	0.6mm	19	93.7849	93.2284	100.943	101.2573	106.886	106.9252	55.8668
tma a1-2	0.6mm	20				83.9428	90.0672	77.0093	1.5789

Supplementary Table 2: The mean H-scores for 1.0 mm TMA cores and the whole sections for 20 samples

Slide	Size	Sample No.	Six Cores	Five Cores	Four Cores	Three Cores	Two Cores	One core	Whole Section
tma a1-2	1.0mm	1	155.6665	152.5979	156.4935	144.0588	155.7493	155.2707	191.0037
tma a1-2	1.0mm	2	79.8836	81.0064	84.4664	90.3637	95.5575	114.023	32.528
tma a1-5	1.0mm	3				7.8144	6.4743	5.7848	176.0478
tma a1-3	1.0mm	4				22.0137	23.0205	24.3386	116.1594
tma a1-4	1.0mm	5	92.1232	71.7371	87.1421	113.3378	166.132	208.8677	50.39
tma a1-5	1.0mm	6			189.9537	182.9382	182.9514	167.754	21.361
tma a1-7	1.0mm	7	72.2262	68.9667	67.4923	63.7773	56.0723	56.9721	77.0994
tma a1-8	1.0mm	8	92.5682	97.2357	100.5648	106.3989	119.4488	162.3106	104.4944
tma a2-7	1.0mm	9			231.018	228.8279	223.4436	238.6425	185.7361
tma a2-9	1.0mm	10				15.9421	20.4182	23.6264	25.1934
tma a1-3	1.0mm	11				38.2633	27.677	33.6516	17.9218
tma a8-3	1.0mm	12	213.0542	206.0902	205.0833	216.209	197.0585	220.1017	191.2527
tma a8-3	1.0mm	13	212.471	217.1499	20.4725	224.5367	207.8591	214.9533	143.0293
tma a8-3	1.0mm	14	254.1115	251.5934	246.6363	242.8242	240.4987	264.5409	214.2186
tma a8-5	1.0mm	15	24.3222	27.0573	23.7146	16.8091	14.6904	21.2312	13.4525
tma a3-3	1.0mm	16	80.431	80.2345	88.5647	97.1733	98.4907	123.7611	73.0539
tma a3-3	1.0mm	17	70.349	69.8949	74.506	77.9585	87.7807	109.8936	44.8392
tma a4-4	1.0mm	18	150.638	135.9344	116.7212	110.781	108.4792	73.7399	115.9934
tma a4-4	1.0mm	19	88.1895	87.7329	96.7179	97.4678	101.2041	97.1	55.8668
tma a1-2	1.0mm	20				77.166	84.4406	80.8713	1.5789

Supplementary Table 3: The mean H-scores for 1.5 mm TMA cores and the whole sections for 20 samples

Slide	Size	Sample No.	Six Cores	Five Cores	Four Cores	Three Cores	Two Cores	One core	Whole Section
tma a1-2	1.5mm	1	154.1588	150.3473	151.3759	148.5799	159.3927	170.6875	191.0037
tma a1-2	1.5mm	2	75.3816	75.1898	77.1198	83.2951	85.2682	98.0083	32.528
tma a1-5	1.5mm	3				6.4681	5.961	6.4324	176.0478
tma a1-3	1.5mm	4				37.9623	27.2255	33.6516	116.1594
tma a1-4	1.5mm	5	86.1948	71.8763	86.7709	11.5157	164.5813	211.7115	50.39
tma a1-5	1.5mm	6			179.7642	174.7685	180.5876	172.2812	21.361
tma a1-7	1.5mm	7	76.0496	71.2202	63.1582	58.963	45.1431	44.7297	77.0994
tma a1-8	1.5mm	8	90.8068	93.1111	97.6727	102.6193	103.8037	129.8955	104.4944
tma a2-7	1.5mm	9			181.6045	164.18	144.559	194.3501	185.7361
tma a2-9	1.5mm	10				15.9742	18.7652	23.6008	25.1934
tma a1-3	1.5mm	11				35.8998	25.573	30.3466	17.9218
tma a8-3	1.5mm	12	208.0691	203.9097	202.1625	218.5194	206.5829	229.307	191.2527
tma a8-3	1.5mm	13	197.8191	204.7383	206.5808	206.32	188.8108	216.129	143.0293
tma a8-3	1.5mm	14	250.6079	246.5114	241.0272	238.0932	233.7054	248.9276	214.2186
tma a8-5	1.5mm	15	25.4611	28.1224	25.2861	17.6418	15.5898	22.1773	13.4525
tma a3-3	1.5mm	16	80.267	80.8588	88.8306	95.2741	95.0777	112.5683	73.0539
tma a3-3	1.5mm	17	74.8564	75.5114	79.0802	81.6948	87.2221	106.0351	44.8392
tma a4-4	1.5mm	18	142.1107	125.9181	114.7151	108.241	111.635	125.7152	115.9934
tma a4-4	1.5mm	19	86.6074	87.452	95.8013	96.0996	99.0087	91.8426	55.8668
tma a1-2	1.5mm	20				80.8029	88.0045	86.1533	1.5789

DISCUSSION

The aim of this study was to find out the optimal number of TMA cores to accurately represent the whole section with IHC analysis in OSCC. The tissue samples were stained with p53 protein marker. In order to assess the immunoreactivity of the cancer cells, H-scores of the cores and whole sections were calculated. This algorithm includes capturing the percentage of tumor cells stained at each intensity level. This score is more representative of the staining of the entire tumor on the section. Although given sections may share the same simple intensity score, there clearly is a difference between a 3+ case with only 10% of the cells staining as compared to a 3+ case where greater than 90% of the cells are staining. This difference is easily picked up using H-Score method. An H-Score is typically calculated for staining of each sub-cellular compartment for both normal and tumor cells using the following formula; H-Score = (% at 0) x 0 + (% at 1+) x 1 + (% at 2+) x 2 + (% at 3+) x 3. Thus, this score produces a continuous variable that ranges from 0 to 300.

In this study, it was observed that from the intra-class correlation and the one sample t-test that one 0.6mm core is sufficient to represent the whole section with a correlation of 0.828. Furthermore, the intra-class correlation shows that increasing the number of cores gives higher levels of association as well as narrower range of 95% CI. Increasing the size of the cores also yields better concordance between the TMA cores and the whole section. However, as the main purpose of TMA is to conserve the tissue available, it is shown that only one core of 0.6 mm is adequate to represent the whole tissue section.

However, the problem of tissue loss is a prominent issue in other similar studies which have used constructed TMA blocks. Gulmann *et al* (2003) reported a loss of 15% of cores in their study (23). Fernebro *et al* (2002) and Griffin *et al* (2003) reported 17% loss of tissue samples (17, 20) whereas Monteiro *et al* (2010) reported the highest,19.8% loss of TMA core samples (21).

Although a single core of 0.6mm is sufficient to adequately represent the whole section, when the sample loss is taken into account, it is suggested that minimum of three cores of diameter 0.6 mm be used. This not only gives a high level of concordance between the TMA and the whole section, it also provides for the event of loss of tissue core. Furthermore, the third core will be able to verify should the first two cores display contradictory results during semi-quantitative scoring.

Normally, TMA cores are constructed from the donor tissue blocks whereas in this study virtual cores were constructed on scanned images of the whole tissue

section and used for analysis. Hence, this may not represent the occurrence of real-life events when using real TMA cores such as the presence of unusable cores due to loss or folding of the tissue. Therefore, in this study, losses of tissue TMA cores due to unusable cores were not accounted for in the analysis. Moreover, in this study, the virtual TMA cores were selected after immunostaining was performed, from areas that exhibited immunostained cells in the whole section. This might have led to some selections bias whereas, in real-life events using TMA, the cores are selected before immunostaining is performed which may result in selection of areas that may not have the immunostaining. It is suggested that this study be carried out with a larger sample size. This study should also be validated using real TMA cores in order to fully depict the real-life implications where the TMA core losses come into consideration in the results. This study can be further extended to include other markers such as Ki-67, p63 to analyze if there is a need for performing such studies for each marker when using TMA with oral cancer tissues.

CONCLUSION

Three TMA cores of 0.6 mm would be the most optimal, as not only is there a very strong correlation with the whole tissue section, the extra core will also be able to act as confirmation if the results of the first 2 cores are in doubt.

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Declaration of conflicts of interest:

The authors have no conflicts of interest to declare.

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