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Research Article

Aurora-A Overexpression Plays a Role in Developing Aneuploidy in a Subset of Oral Squamous Cell Carcinoma

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Abstract

Introduction: Aurora-A is a member of the mitotic serine/threonine kinase family, and is an essential protein expressed during the cell cycle of many types of cells. Since its expression differs according to localization and time of activation, it could provide an important cell marker in cancer.

Materials and Methods:A total of 125 known Oral Squamous Cell Carcinoma (OSCC) histopathological records were obtained and included in this study. The cases varied in location of the primary site and stage of the lesion. All cases were stained for immunohistochemical analysis of Aurora-A, for protein expression profile and for ploidy analysis by use of image cytometry. Independent sample tests were carried out to evaluate the reliability of the analysis.

Results: Aurora-A expression was observed in 88.8% of OSCC and was observed to be higher than in fibroepithelial polyps (FEP). Of the aneuploidy OSCC, 75.3% showed medium to high Aurora-A expression versus 31.6% of diploid tumors.

Discussion: Our findings suggest that Aurora-A is ectopically over-expressed in OSCC throughout the cytoplasm as opposed to being expressed in the centrosomes during normal mitosis. However, it appears that Aurora-A is at best a weak oncogene that acts in concurrence with other factors to induce carcinogenesis.

Keywords: Aurora-A, licensing proteins, OSCC, cell cycle

Material and Methods

Patients and Archival Tissue Samples

Ethical approval was obtained from the Joint Research and Ethics Committee in the Eastman Dental Institute (EDI) and Hospitals for patients diagnosed with primary OSCC who had incisional or excisional biopsies or surgeries and fully abides with the World Medical Association Declaration of Helsinki.. Histopathological records of the EDI and Department of Pathology of the University College London, UK were used to identify subjects to be included in the study. All samples had originally been fixed in a 4% dilution of 10% formalin saline concentrate, processed and embedded in paraffin wax. Exclusion criteria included patients who underwent pre-operative radiotherapy or chemotherapy. Patients' notes were used to retrieve the following information: date of birth, gender, and site of primary tumour, date of diagnosis, differentiation, date and type of surgery, TNM staging and follow-up periods.

Three and five-year survival data were retrieved from the Thames Cancer Registry Office. Histologic diagnoses were sub-

jectively classified according to the Broder's criteria using a modification of his classification system [18]. The TNM system was used to classify tumours clinically as follows: Stage I, T1N0M0; Stage II, T2N0M0; Stage III, T3N0M0, or any T with N1 M0; and Stage IV, any T with N2M0, N3M0, or any N with M1.

In addition, grading of the mode of invasion was objectively carried out according to Odell et al. [19] as follows: Grade 1; well delineated borderline; Grade 2; infiltrating cords, bands and strands; Grade 3; small groups or cords of less than 15 cells; and Grade 4; marked extensive cellular dissociation in small groups or single cells. For reliability of histopathological classification, 10% of randomly selected cases were re-assessed by two different pathologists (Bill Barrett and Garth Thomas). As a control group, 18 fibroepithelial polyps were identified from the records. Patients' notes were used to gather the following information: date of birth, gender and date of diagnosis.

Immunohistochemistry

Paraffin blocks were available for all patients; blocks contained tissue peripheral to the tumour. Sections from the chosen blocks were serially cut, prepared, deparaffinized, rehydrated and antigen retrieval was done Next, immunostaining was automated using a Bond immune stainer (Vision Biosystem). Non-specific activity of endogenous peroxidase was quenched with peroxidase blocking solution for 5 minutes. The slides were then washed (using a wash solution for this and subsequent washes) 3 times for 2 minutes and drained. Subsequently, the primary antibody was incubated for 30 minutes, washed 3 times for 2 minutes and drained. The best working antibody dilution in this series was Aurora-A 1/40 (A: TBS). Then, a post primary antibody was incubated for 20 minutes and washed 3 times for 2 minutes. After that, a polymer-based secondary antibody and peroxidase molecules were incubated for 20 minutes, washed 3 times for 2 minutes and drained.

Immunostaining used DAB for 10 minutes with subsequent washing in deionised water 3 times for 1 minute with draining. Next, an enhancer was applied for 5 minutes, washed with deionised water 3 times for 1 minute and drained. i.e. it enhances the chromogen's colour. Finally, the slides were counterstained with Mayer's haematoxylin for five minutes, washed with deionised water, drained, dehydrated, cleared, mounted For quality control, appropriate tissue sections from the colon were used as positive controls; these underwent identical sample preparation.

Protein Expression Profile Analysis

The Labelling Indices (LI) of each tumour were calculated to analyse the Aurora-A protein expression. Initially, a pathologist used the Olympus BX51 microscope to scan all slides under low power and identify a representative area. Next, images from the selected area were captured with a CCD camera and Analysis software (SIS, Munster, Germany) from the invasive front towards the periphery (Kodani et al. 2001) at 10x magnification for orientation purposes. A tumour axis was determined and another 2 to 5 images were taken following this axis at 20x magnification. These 20x magnification images were enlarged to 30x magnification for counting. Then, images were printed for quantitative analysis. Analysis was done without knowledge of the clinico-pathological records. Tumour cells were counted at the chosen fields ignoring any stromal or inflammatory cells. The presence of any nuclear staining was considered positive, and the LI was calculated by dividing the number of positive cells by the total number of counted cells.

In addition, the intensity was assessed semi-quantitatively as follows: ++, high expression was detectable within the lesion; +, moderate expression was detectable within the lesions; +/-, expression was weakly detectable in part of the lesions; and -, expression was not detectable within the lesions. This semiquantitative scoring was adapted from Tanada et al., however we modified it for statistical significance [20].

Image Cytometric Analysis for Ploidy

The DNA content was analysed using the method of Sudbo et al. [21]. The most representative area of the lesion was outlined in each slide. The selected areas were identified in the blocks and marked. These areas were micro-dissected, and 1-6 sections that were 50-micron thick were cut and placed in Falcon tubes. The sections were cleared by adding 10 ml of xylene for 10 minutes with removal of supernatant (repeat twice). They were then hydrated by adding 10 ml of 100% and 95% ethanol for 5 minutes each with 1ml of deionised water for 5 minutes (the last step was repeated twice). Next, 10 ml of cold PBS was added for 5 minutes.

Enzymatic digestion was achieved by adding 2 ml of protease XXIV for 2.5 hours in a shaker water bath at 37ºC. After digestion, 3 ml of chilled PBS was added, filtered through a nylon mesh into 15ml Falcon tubes and centrifuged for 5 minutes at 1500 rpm. The pellets were re-suspended in 3 ml of fresh PBS. Then 100-200 µl of the re-suspended pellets was cytospun in a cytocentrifuge at 1500 rpm for 5 minutes and fixed in 4% formalin overnight. The Next day, the slides were rinsed in tap water, placed in 5 M HCL for 1 hour for hydrolysis, rinsed in distilled water and stained with Schiff's reagent for 2 hours (the last step is done in the dark). Subsequently, the slides were washed in running tap water for 10 minutes and dehydrated in increasing concentrations of ethanol (70%, 95% and 100%) for 10 seconds each. Finally, the slides were mounted and scanned using an Axioplan 2 imaging microscope. The nuclei were analysed with Fairfield imaging software, Kent, UK. The Lymphocytes were used as internal controls to guide histogram scaling.

Histograms were interpreted based on Sudbo's classification of aneuploidy [21]. Diploid lesions were defined by the presence of only one 2c peak or if the number of nuclei in 4c did not exceed 10% of the total number of epithelial nuclei or if the number of nuclei with more than 5c DNA content was less than 1% of the total number of nuclei. The lesions were classified as tetraploid when their G0/G1 (4c) peak was present together with its G2 peak (8c) or when the fraction of the nuclei in the tetraploid region exceeded 10% of the total number of nuclei. Lesions were classified as aneuploid by the presence of non-euploid peaks or if the number of nuclei with DNA content was greater than 5c or 9c exceeded 1% [21].

Statistical Analysis

Independent sample tests to evaluate equality of means were carried out for reliability of analysis, and these showed no significant differences. The expression profiles of Aurora-A were then compared to both OSCC and FEP using student's t test and the chi-square test. Association between Aurora-A expression and other factors were assessed using the Mann-Whitney U test, the Kruskal-Wallis test, the Jonckheere-Terpstra test, and Spearman's rank correlation coefficient test. All statistical results were two-sided. The expression levels of Aurora-A in relation to overall survival periods and disease free survival periods were tested with Kaplan-Meier analysis using the log-rank test. This statistical analysis used SPSS 12.0.1 for Windows (SPSS, Inc., Chicago, IL).

Results

Demographic Data

Of 172 HNSCC specimens collected, 125 OSCC cases were suitable for immunohistochemical analysis. Here, 9, 12, and 26 cases were excluded because of exposure to radiotherapy or photodynamic therapy, extra oral origin (8 from the oropharynx, 2 from the maxillary antrum, 1 from the scalp, and 1 from the parotid gland), and insufficient tissue or proper orientation for analysis, respectively. The immunohistochemical samples included 35 biopsies and 90 resections from 80 males and 45 females with a male to female ratio of 1.8:1. The age range was 27-96 years with a median age of 60 years. The primary site of the OSCC included 54 cases from the tongue (22 from the lateral border and the rest were not specified), 39 from the alveolar mucosa, 21 from the floor of the mouth, 6 from the buccal mucosa (1 from the commissures), 4 from the lower lip, and 1 from the hard palate (Table 1).

Clinical Features	Total	Clinical Features	Total
All cases		125	Stage
Gender	I	12	
Male	80	II	9
Female	45	III	3
Unknown	0	IV	58
Age	Unknown	43	
≤ 60 years	62	Tumour size	
≥ 60 years	62	T1+T2	36
Unknown	1	T3+T4	50
Primary tumour site		Unknown	39
Tongue	54	Lymph node metastasis	
Alveolar mucosa	39	No	64
Floor of the mouth	21	Yes	41
Buccal mucosa	6	Unknown	20
Others	5	Three-years survival	
Unknown	0	Survived	31
Phenotype		Did not survive	48
Conventional	113	Unknown	46
Verrucous/papillary	9	Five-years survival	
Adenosquamous	2	Survived	14
Basaloid	1	Did not survive	51
Unknown	0	Unknown	60
Histopathologic differentiation		DNA content	
Well	26	Diploid	19
Moderate	71	Aneuploid	85
Poor	28	Tetraploid	0
Unknown	0	Unknown	21
Invasive front		AA intensity expression	
Pushing	9	No expression	14
Bands	53	Weak expression	25
Cords	38	Medium expression	35
Diffuse	25	High expression	51
Unknown	0	Unknown	0

Table 1: Clinicopathological and outcome parameters in OSCC.

The 18 fibroepithelial polyps were all suitable for analysis. Five were males, and the remaining 14 cases were females. The age ranged from 18 to 78 with a median age of 44.5 years.

Histologicpathologic, Clinical Staging and Clinical Outcome Data

From the 125 OSCC, 113 cases were conventional OSCC, 9 were verrucous, 2 were adenosquamous and one was basaloid. Twenty-six cases were well differentiated, 71 were moderately differentiated, and 28 were poorly differentiated. In terms of tumour invasiveness, 9 were classified as having pushing fronts, 53 had bands at the front, 38 had cords at the front and 25 had a diffuse front.There were 82 cases with TNM staging information includ-

ing 41 with lymph node metastasis. Information on follow up periods was available over a three-year survival period in 79 cases (63.2%): 31 survived and 48 did not. There was 5-year survival data from 65 cases (52%): 14 survived and 51 did not (Table 1).

Aurora-A Expression in Fibro Epithelial Polyps and OSCC

A total of 94.4% (17/18) of the FEP showed expression of Aurora-A. This expression was demonstrated in the cytoplasm of a small number of basal cells of the FEP (Median LI; 3.4%) (Figure 1). The limited expression of Aurora-A was further complimented by the difference of its LI versus other proliferative markers such as Mcm2, Ki67 and Geminin (not published results) [Median: Mcm2, 41.6%; Ki67, 24.2%; Geminin, 5.5%; Aurora-A, 3.4% (Figure 2. The FEP showed were predominantly (55.6%) undetectable (-, 1 FEP from a total of 18 FEP specimens) or weak (+, 9/18) expression of Aurora-A in the cytoplasm. We noted that 44.4% (8/18) of the cases had medium to high expression. Furthermore, all analysed FEP had normal diploid DNA content.

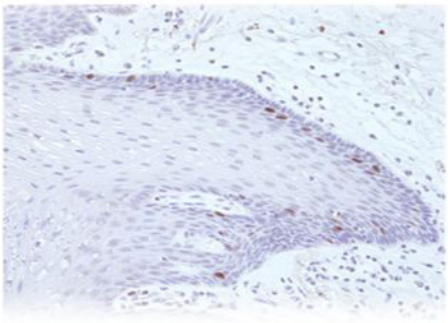


Figure 1: Immunohistochemical staining of fibroepethelial polyps for Aurora-A (positive cells stained brown). Aurora-A expression was predominantly cytoplasmic and at the basal layers of these specimens. Original magnification x 400

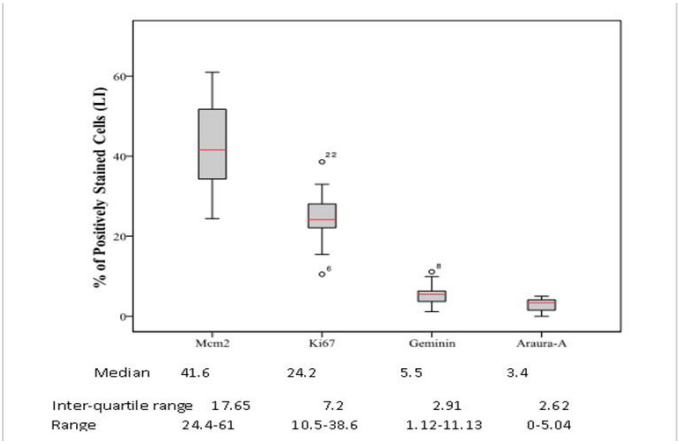


Figure 2: The median (solid red line), interquartile range (boxed), and range (enclosed by lines) of Mcm2, Ki67, Geminin and Aurora-A expression are shown in oral fibroepethelial polyps (outlying cases are shown by isolated points designated by the case number). The median of Aurora-A was significantly less than the rest of the markers (paired t test, P< 0.001 each).

However, Aurora-A's expression was noted in 88.8% (111/125) of OSCC with a diffusely distributed expression in the cytoplasm at the peripheral portions of the OSCC nests. They were sometimes found scattered throughout some tumours (Figure 3). The diffuse cytoplasmic distribution of Aurora-A might imply that this protein is ectopically overexpressed in OSCC rather than its normal localisation in the centrosomes and mitotic spindle. In addition, Aurora-A was expressed in proliferating cells in OSCC as seen by its intensity association with Ki67 expression (Jonckheere-Terpstra test, P= 0.02) (Figure 4). However, only a subset of cycling cells expressed Aurora-A as indicated by its low LI versus other proliferative markers such as Mcm2, Ki67 and Geminin (data not shown) [Median: Aurora-A, 4.5%; Mcm2, 71.9%; Ki67, 55.2%; and Geminin, 19.5% (paired t test, P < 0.001 each)] (Figure 5).

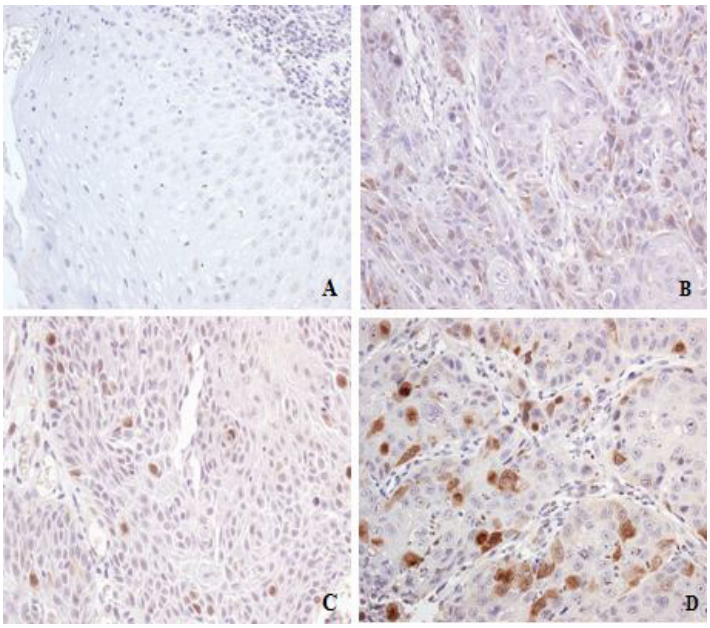
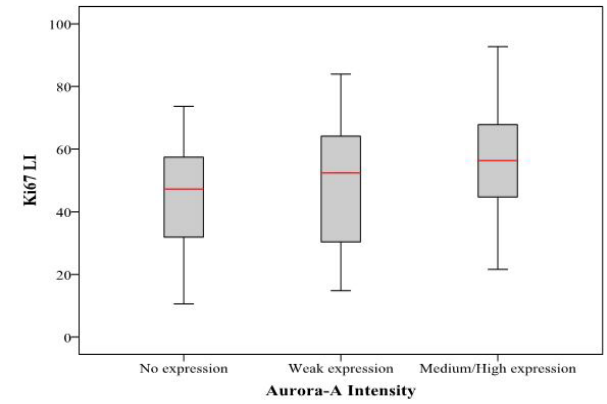
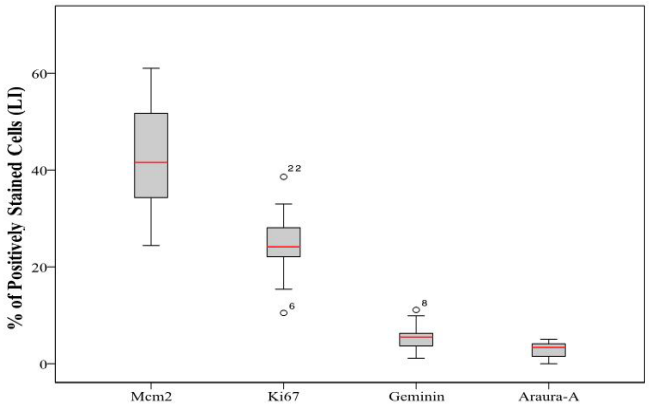


Figure 3: Immunohistochemical staining of OSCC for Aurora-A (positive cells stain brown). Aurora-A staining is diffusely distributed in the cytoplasm, at the peripheral portions of the cancer nests (A) and sometimes found scattered throughout some tumours (B). Original magnification was x 100 and x 400 (the enlarged images).



Median	47.3	52.4	56.4
Inter-quartile range	26.6	35.6	23.2
Range	10.60-73.60	14.80-83.90	21.60-92.70

Figure 4: Median, interquartile range, and range of Kki67 according to intensity of Aurora-A expression is shown in OSCC. The median of Ki67 is significantly higher in cases with high Aurora-A intensity (Jonckheere-Terpstra test, P= 0.02).



Median	72	55.25	19.15	4.57
Inter-quartile range	22	21.98	10.85	6.98
Range	27.40-97-70	10.60-92-70	2.70-60.60	0-26.80

Figure 5: Median, interquartile range, and range of Mcm2, Kki67, Geminin, and Aurora-A expression are shown in OSCC. The median and interquartile range of Aurora-A are significantly less than those of the rest of the markers (paired t test, P < 0.001 each).

The OSCC specimens had frequently (68.8%, 86/ 125) high (++, 51/ 125) or medium (+, 35/125) expression in the cytoplasm of tumour cells. This elevated intensity might represent ectopic overexpression of Aurora-A in cell-cycle phases other than the normal G2/M phases. This might implicate Aurora-A in OSCC carcinogenesis. Moreover, the higher Aurora-A expression in OSCC relative to FEP (Chi-square test, P= 0.025) might further implicate it in OSCC carcinogenesis. Approximately, 31% of cases had weak expression (+/-, 25/ 125) or no detectable expression (-, 14/ 125).

Relationship between Aurora-A expression in OSCC, aneuploidy, histopathologic, and outcome parameters

Next, we used image cytometry of DNA content to investigate the link between Aurora-A expression and DNA content. A nuclear monolayer was prepared for 125 OSCC subjects. Of these, 103 OSCC were suitable for analysis and histogram interpretation .Figure 6 shows histograms and their interpretation of representative OSCC. The number of nuclei analysed per case ranged from 354-1053 nuclei (median 809) with a median of 10 lymphocytes as internal controls. 19 tumours had diploid DNA content, and 85 cases had aneuploid DNA content. Of the 85 aneuploid OSCC, 64 (75.3%) had medium to high Aurora-A expression versus the 31.6% of the diploid tumours with moderate to high expression (Chi-square test, P= 0.01) (Figure 7). Thus, ectopic overexpression of Aurora-A might play an important role in inducing aneuploidy

and tumour formation in OSCC. However, 31.6% (6/19) of the diploid tumours and 44.4% (8/18) of the diploid fibroepithelial polyps had ectopic overexpression of Aurora-A (medium to high expression). This might imply that Aurora-A ectopic overexpression does not independently induce aneuploidy and tumourgenesis in OSCC

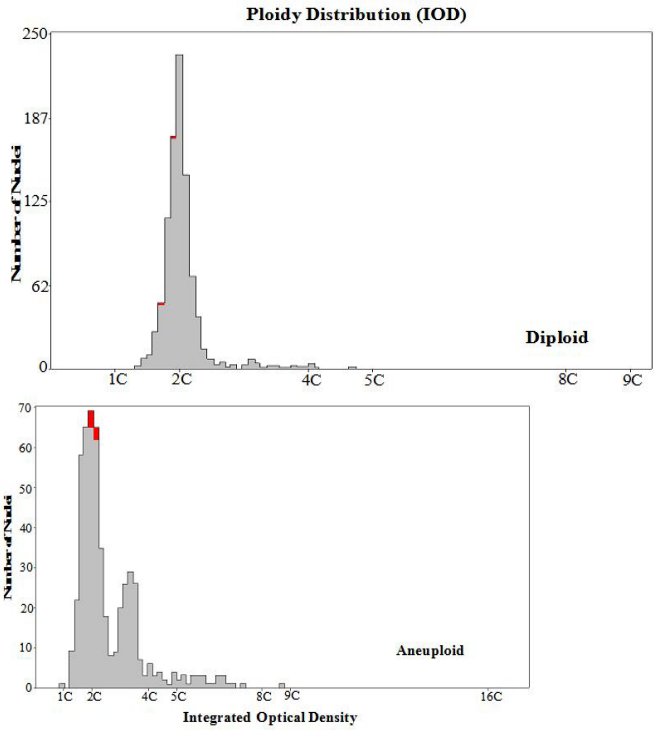


Figure 6: DNA histograms generated by measurements of the nuclear DNA content of Fuelgen-stained OSCC cells (grey) of diploid and aneuploid tumours. The histograms were scaled on the basis of DNA content of a diploid standard (lymphocytes, red). Histogram interpretation for DNA content was based on Sudbo's classification for aneuploidy.

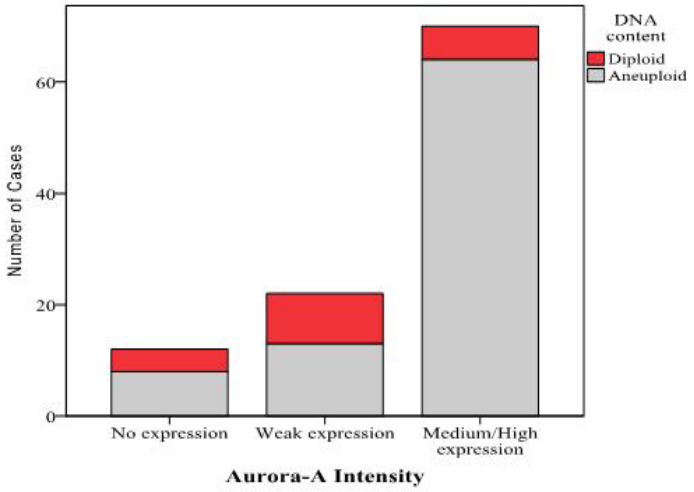


Figure 7: Aurora-A ectopic overexpression and its relation with aneuploidy in OSCC. Among the 85 aneuploid OSCC, 64 cases had moderate to high Aurora-A expression; while only 6 cases from the 19 diploid OSCC had moderate to high expression (Chi-square test, P= 0.01). Thus, it is possible that Aurora-A ectopic overexpression induces aneuploidy in OSCC.

Aurora-A expression measured by LI did not correlate with any histopathologic parameters, clinical outcome parameters or DNA content. Accordingly, there was no association between Aurora-A expression and differentiation and stage (Jonckheere-Terpstra test, P= 0.315, P=0.59 and P=0.85 respectively). There was no association between Aurora-A expression and gender, age or DNA content (Mann-Whitney U test, P=0.302, P=0.94, P=0.295 and P=0.752 respectively). There was no correlation between Aurora-A expression and lymph node metastasis (Spearman's correlation coefficient, P= 0.688). Additionally, Aurora-A expression was not a good predictor of five-year survival rates (Log-Rank test, P= 0.17) (Table 2 and 3).

Discussion

Oral cancer is the eighth most common tumour worldwide, and approximately half of all patients die within five years [22,23] This poor survival is attributed to late-stage survival when lymph node metastasis and/or invasion of surrounding tissues have occurred [24]. Therefore, there is a need to understand the molecular mechanisms of oral cancer,particularly OSCCto enable new approaches for diagnosis and treatment. Cancer development is partly due to alteration in the expression and/or mutations of cell-cycle regulators. Abrogation of mitosis and its checkpoints have been reported to result in abnormalities in nuclei and chromosomal segregation with subsequent aneuploidy development [25].

In our study, we showed that 88.8% (111/125) of the OSCC expressed Aurora-A, and the majority (68.8%, 86/125) were found to overexpress it diffusely throughoutthe cytoplasm. This diffuse overexpression suggests that Aurora-A is ectopically overexpressed in OSCC throughout the cytoplasm rather than its normal localisation in the centrosomes and mitotic spindle. Additionally, the high Aurora-A intensity seen in most cells expressing this protein suggests that it is expressed independently of the cell-cycle phases rather than the typical increase seen in G2/M phases (cell-cycle phase dependent). This spatial and temporal ectopic overexpression of Aurora-A promotes oral tumourgenesis via excessive phosphorylation of normal substrates and abnormalphosphorylation of cytoplasmic proteins (involved in oncogenesis) or proteins expressed in other phases of the cell cycle [7]. Aurora-A was overexpressed in 55.6% of FEP, which possibly suggests that Aurora-A is a weak oncogene and that other defects cooperate with it to induce oral carcinogenesis.

To the best of our knowledge, this was the first immunohistochemical report to show that primary OSCC overexpress Aurora-A (67.7%). Tatsukaet al. reported Aurora-A gene amplification (36.36%, 4/11) and mRNA overexpression (100%, 11/11) in tongue and gingival carcinoma, but did not analyse the protein expression [14]. Furthermore, Aurora-A was expressed in only a subset of cycling cells, which might suggest that Aurora-A is only an indicator of cancer cell proliferation relative to other existing proliferative markers.

There was no association between Aurora-A overexpression and clinic pathological details or outcome parameters. These finding weresupported by other reports of breast tumours [26]. One reason might be that the Aurora-A expression analysis by IHC is not the most effective method of analysis [27]. Other methods such as gene amplification and mRNA might be better atcorrelating with clinical and histopathologic parameters. This might be because when the sample is subdivided according to different histopathologic parameters, the subgroups become small, and the statistically significant differences become less obvious. It is also possible that Aurora-A overexpression might be found in patients with poor prognosis, but adjuvant treatments altered the natural history of the diseaseleading to no survival differences. Thus, a correlation might still exist, but this study was too small to detect it. Moreover, recent reports support the involvement of Aurora-A in early events in tumour carcinogenesis; thus, it is possible that Aurora-A overexpression could be an initiation marker rather than a prognostic marker.

Although a high level of Aurora-A expression appeared to be common in OSSC, 24.7% (21/85) of the aneuploid tumours showed weak or no expression of Aurora-A. This suggests that in a fraction of OSCC, other genes are associated with the development of anueploidy. Thus, Aurora-A overexpression most probably acts together with other gene products involved in chromosomal segregation to induce aneuploidy in OSCC. One good candidate is inactive p53 tumour suppressor protein. This has been shown to correlate with Aurora-A overexpression in hepatocellular carcinoma when mutated (TP53). This observation provides clinical evidence that Aurora-A overexpression and TP53 cooperate in tumour formation [28].

This study suggests that Aurora-A protein overexpression in OSCC cooperates with other oncogenes to disrupt the signalling cascade that regulates equal segregation of chromosomes leading to pronounced aneuploidy. Future studies in Aurora-A regulators could help explain the mechanisms underlying these processes. The results might enable us to develop new approaches to early diagnosis and treatment.

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