Induction of lymphomas on implantation of human oral squamous cell carcinomas in nude mice

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Cancer cells from five oral cancer patients and pleomorphic adenoma cells from one individual were inoculated as single cell suspension into subcutis of 30 Swiss nude mice and tail vein of additional 30 mice. Further, tumor tissue pieces from three oral cancer patients were xenografted sc in 18 nude mice, and 10 mice were kept as controls. In animals implanted with tumor pieces, 7/18 (39%) mice, developed squamous cell carcinoma at the site of inoculation within 8-15 days, while tumors were not observed in mice inoculated with single cell suspension, up to 60/90 days. In 8/68 (12%) mice, white foci were observed in several tissues, with hepatomegaly and splenomegaly noted in 27/68 (39%) mice. Histopathological examination of various tissues revealed presence of large cell lymphoma in several organs in 14/68 (21%) mice. No regional or distant metastasis of the implanted oral tumor cells was detected. Mice injected with cells from pleomorphic adenoma, also demonstrated large cell lymphoma in 2/10 (20%) mice, whereas none of the 10 control animals showed any gross abnormalities or microscopic abnormalities in several organs. 2/16 (12%) lymphomas exhibited positive reaction with mouse B cell antibodies illustrating the murine origin of the lymphomas, and these were immunophenotyped as B cell lymphomas. The lymphomas were also examined with mouse T cell antibodies and none reacted positively with the mouse T cell antibodies. The lymphomas also failed to react with human T cell, B cell and human Leucocyte common antigen (LCA) antibodies, indicating that the induced lymphomas were not of human origin. The tumor specimens from seven of eight oral cancer patients and the pleomorphic adenoma patient induced lymphomas in nude mice. Thus it appears that xenografting oral tumor cells into nude mice may cause induction of the murine lymphomas, and this needs further investigation.

In India, a high incidence of oral cancer is noted and this is attributed to the prevalent habit of tobacco chewing and bidi smoking¹. Despite advances in therapeutic and reconstructive modalities, oral cancer still represents an important cause of morbidity and mortality in India². With a view to understand oral carcinogenesis and consequent prediction of prognosis, and informative decision making for appropriate therapy, molecular lesions have been examined in the multistep progression of oral cancer. Previous studies investigating primary oral tumor tissue of long-term (10-30 year) habitual tobacco chewers in India have demonstrated tumor associated oncogene (myc/ras/EGF-R) activation via amplification, rearrangement, point mutations, and loss of allelic heterozygosity in majority (>90%) of patients³⁴. These oral tumor tissues also showed inactivation of tumor suppressor gene p53 by allelic loss and mutations⁵⁶. Further, a potent transforming oncogene, other than myc/ras/EGF-R has been cloned from primary oral tissue and subjected to nucleotide sequencing of the gene⁷.

Although the prognosis and clinical outcome for patients with oral cancer is largely dependent on the stage of the disease, the biological characteristics of these tumors are often variable resulting in divergent clinical disease course, despite similar or identical staging. Tumor pathology and histological differentiation do not reflect the biological behaviour of the oral cancer cells⁸. To study oral tumor cell biology, studies investigating biological models are essential. Transplantation of human tumors into immunodeficient athymic nude mice is an important experimental approach to study the biology of human oral cancer, with application in planning treatment strategies. The cancer xenografts grown subcutaneously in athymic nude mice, are...
morphologically, biochemically and biologically similar to the original tumors. The aim of the present study was to determine the biological and metastatic behaviour of oral tumor cells, following inoculation of surgical tumor specimens into the subcutis and tail vein of nude mice.

Materials and Methods

Tumor specimens—Oral cancer and pleomorphic adenoma tissue specimens were obtained from nine patients (7 males, 2 females), aged 17 to 65 years, on admission for surgery to the Tata Memorial Hospital, Mumbai, India. The eight tumors were histologically confirmed as oral squamous cell carcinomas (OSCC) and were from the following subsites: buccal mucosa (4 patients), lower alveolus (2 patients), tongue and lower lip (1 each) and a single pleomorphic adenoma. The eight oral cancer patients were habitual tobacco chewers while the patient with pleomorphic adenoma was a seventeen year old boy with no tobacco habits.

Mice—Athymic Swiss nude mice (88) obtained from the animal facility of National Institute of Nutrition, Hyderabad, India (6-8 week old, 45 males and 43 females) were used. The mice were maintained in a laminar flow isolator (Harlon, England), under specific pathogen-free conditions and handled in sterile conditions.

Preparation of tumor cell suspension for in vivo injections—The oral cancer tissues were extensively washed with 0.1M phosphate buffered saline pH 7 (PBS), containing gentamycin (50 μg/ml). The tumour tissues were minced in PBS, filtered through sterile gauze to obtain single cell suspension, and cells with greater than 90% viability as assessed by trypan blue dye exclusion, were used for in vivo injections.

Subcutaneous inoculation of tumor cells/tissue—Tumorigenicity and metastatic potential of oral tumor cells were assessed subsequent to sc implantation of tumor cells/tissue in the lateral thoracic region, in two groups of animals. A group of 30 nude mice were injected with single cell suspension (1x10⁶ tumor cells/0.2 ml PBS) sc from five oral squamous cell carcinoma (OSCC) patients, and one pleomorphic adenoma patient. In the second group, 1-2 mm³ tumor pieces from 3 oral cancer patients were implanted sc in an incision made in the lateral thoracic region in 18 recipient nude mice. The incisions were closed with sterile sutures. PBS (0.2 ml) was injected sc into 5 nude mice as controls for this aspect of the study. Implantation sites were examined twice a week. The nodules (<1 cm³ diam.) were resected under aseptic conditions, after giving 2.5% avertin, and the skin incisions sutured. The mice in both the groups were killed 90 days post-inoculation of cells, or sooner if they became moribund.

Intra venous injection of tumor cells—To study experimental metastasis, 1x10⁶ tumor cells/0.2 ml PBS, from 5 oral cancer patients and one pleomorphic adenoma patient, were injected into the lateral tail vein of 30 nude mice. Five nude mice were injected iv with 0.2 ml PBS, as controls in the study group. The mice were monitored daily, and killed 60 days post-inoculation of cells, or sooner if they became moribund.

Histological studies—The excised nude mice tumors and visceral organs including lung, liver, kidney, spleen, intestine, lymph nodes and any other organ showing gross abnormality were fixed in 10% neutral buffered formalin and processed for histological examination. Tissue paraffin sections (5 μm thick) were stained with hematoxylin and eosin (H and E) and observed under light microscope.

Immunohistochemical analysis—The paraffin sections were subjected to immunohistochemical analysis as previously described, with the following antibodies:

Anti-mouse B cell antibodies: B220, 1:50 (Pharmingen) and CD21, 1:50 (gifts from Dr R Cornall, John Radcliffe Hospital, Headington, UK); Anti-mouse T cell antibodies: CD45R/CT1, 1:20 (Pharmingen); CD3e, 1:50 (Pharmingen) (gift from Dr K. Vora, Jefferson Institute, USA); Thy1, 1:50 (gift

Fig. 1—Photomicrograph of oral squamous cell carcinoma growing sc in nude mice. The tumor exhibits the morphology of a moderately differentiated oral squamous cell carcinoma. H and E; scale bar = 140 μm (x500).
from Dr. S. Rath, NII, India); Anti-human B cell antibody: CD20, 1:50 (DAKO); Anti-human T cell antibody: CD3, 1:50 (DAKO); and Anti-human leukocyte common antigen (LCA): 1:50 (DAKO). The slides were counterstained with hematoxylin, mounted in DPX and observed under light microscope.

Results

Subcutaneous tumors—Single cell suspension of OSCC from five individual patients and one pleomorphic adenoma, did not form primary tumors up to 90 days at the site of inoculation when injected sc in 30 nude mice. In contrast, 7 of 18 (39%) nude mice formed progressively growing tumors at the site of tissue implantation, 8-15 days post-inoculation on sc implantation with oral tumor tissue pieces from three OSCC patients. The tumors were excised after reaching a size of ~1 cm, and histological examination of these xenografts confirmed the presence of OSCC, resembling the original patient tumor (Fig. 1).

Lymphoma induction—Hepatomegaly and splenomegaly were noted as abdominal enlargement within 15-30 days in mice inoculated with oral tumor cells/tissue and confirmed on autopsy, in 27/68 (39%) mice, as also in 2/10 (20%) mice injected with pleomorphic adenoma cells (Fig. 2a and c). Besides, 8/68 (12%) nude mice inoculated with oral tumor cells or tissue and 2/10 (20%) mice injected with pleomorphic adenoma cells also exhibited white disseminated foci on several visceral organs including

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Fig. 2—(A) Gross view of nude mice showing prominent multifocal liver nodules and severe hepatomegaly. 10 weeks post sc implantation of human oral tumor tissue pieces. (B) Photomicrograph of the liver foci reveals an infiltrating large cell lymphoma. H&E; scale bar = 224 μm (x312.5). (C) Gross view of the organs of nude mouse inoculated with oral tumor cells following iv inoculation, reveals multiple tumor foci on liver and kidneys, in addition to submaxillary lymph node and spleen enlargement. (D) Photomicrograph of the nude mouse kidney with nodules reveals lymphoma infiltrating the interstitial tissue. H&E; scale bar = 224 μm (x312.5).
liver, spleen and kidney (Fig. 2a and c; Table 1). On the other hand, visceral organs of the ten control mice were morphologically and histopathologically normal.

Microscopic examination of the formalin fixed tissues of nude mice inoculated with oral tumor cells/tissues revealed the presence of large cell lymphoma according to Pattengale’s classification\(^{16}\), in 14/68 (21%) mice. Similarly, 2/10 (20%) mice inoculated with pleomorphic adenoma cells, also exhibited the presence of large cell lymphoma (Table 1). Moreover, all the visceral organs exhibiting white foci, demonstrated lymphoma cells on microscopic examination. The lymphomas occurred predominantly in the liver (Fig. 2b) and spleen of the mice. In addition lymphomas were detected in kidney (Fig. 2d), intestine and ovary. It is noteworthy, that tumor cells/tissues from the eight out of nine patients with OSCC and the single pleomorphic adenoma were capable of inducing large cell lymphoma in the nude mice. However, no lymphomas were detected in 10 control mice tissues. Collated results from representative individual experiments measuring

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<th>(\text{TNM classification})</th>
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Abbreviations

All the OSCC patients were habitual tobacco chewers. Patient No.6 with PA was not a tobacco user.

10 control mice: injected 0.2 ml PBS alone, iv in 5 mice and sc in 5 mice
Fig. 3—Photomicrograph illustrating immunohistochemical analysis of mouse and human tissues, using T cell and B cell antibodies. (A) Nude mouse liver lymphoma exhibiting positive reaction with anti-mouse B cell antibody (B220). (B) Nude mouse lymphoma of the kidney demonstrating positive reaction with anti-mouse B cell antibody (B220). (C) Nude mouse kidney lymphoma showing negative reaction with anti-mouse T cell antibody (CT1). (D) Nude mouse fetal thymus (positive control) showing positive staining with anti-mouse T cell antibody (CT1). (E) & (F) Human lymph nodes showing positive staining with anti-human B cell and T cell antibodies (CD20 & CD3) respectively. (G) & (H) Nude mouse lymphomas of the liver and kidney showing negative reaction with anti-human B cell and T cell antibodies respectively (CD20 & CD3). Scale bar = 224 μm (A, B, C, D, G, H) (×312.5); scale bar = 350 μm (E, F) (×200).
lymphoma induction by various tumor cells after sc and iv implantation into nude mice, are presented in Table 1.

Metastasis—Microscopic examination of lymph nodes and several organs like liver, lung, spleen, kidney, intestine and ovaries showed the absence of regional or distant metastasis of the inoculated tumor cells/tissues (iv/sc) in the 78 nude mice. However, lymphomas were observed microscopically in several tissues in 21% mice inoculated with OSCC, as mentioned in the earlier section.

Immunohistochemical analysis—Immunohistochemical staining of the lymphomas with anti-mouse T cell antibodies (CD45R/CTI, CD3e,THY1), anti-mouse B cell antibodies (B220, CD21), anti-human B cell antibody (CD20), anti-human T cell antibody (CD3), and anti-human leucocyte common antigen (LCA) revealed the absence of human T cell and B cell antigens and leucocyte common antigen, indicating that the lymphomas were not of human origin. Fig. 3e and f illustrate positive staining of human lymph nodes (used as positive controls) with human B cell (CD20) and T cell (CD3) antibodies. 2/16 (12%) lymphomas from 78 mice, demonstrated positive reaction with anti-mouse B cell antibody (B220), identifying the lymphomas as of B phenotype of murine origin. Fig. 3a and b illustrate nude mice liver lymphoma and kidney lymphoma respectively exhibiting positive reaction with anti-mouse B cell antibody (B220). The lymphomas, however did not react with any of the anti-mouse T cell antibodies (CT1,CD3e,THY1) (Fig. 3e). Fig. 3d illustrates positive staining of fetal thymus of Balb/c mouse (used as positive control) with mouse T cell antibody (CT1). Fig. 3g and h demonstrate negative reaction of nude mouse liver and kidney lymphomas with anti-human T cell and B cell antibodies respectively.

Discussion
Model studies are indispensible for the development of lead concepts and working hypothesis for the natural history of cancer. The present study was aimed at investigating the biological behaviour of primary human oral cancer in a nude mouse model. It was observed that single cell suspension of 1x10^6 cells/mice inoculated sc in nude mice, did not form tumors at the site of inoculation within 60-90 days. However, intact tumor tissue implanted sc, demonstrated tumors with histopathological identity to the original tumor, within 8-15 days in 39% of nude mice. Thus, tumor tissue implants were tumorigenic, within a short period of 15 days, in comparison to cell suspensions. Metastasis was also checked in distal organs, after a period of three months post-inoculation of the cells and noted absence of regional or distant metastasis in any of the organs. Fu et al. has earlier demonstrated, that cell suspensions may not express metastatic potential of the original tumor, as compared to tumor tissue implanted. Sharkey et al. reported a low incidence (1.7%) of metastasis in various organs during a 30 month period, using 10^6 cells from different human tumors transplanted in 1000 nude mice. On the other hand, Kawashiri et al. observed a high incidence of regional and distant metastasis on injection of two OSCC cell lines when injected directly in the tongue as an orthotopic site.

It was interesting that although distant metastasis of the inoculated primary tumors was not observed, induction of lymphomas in the nude mice was observed. Several viscera, predominantly spleen and liver, showed foci of large cell lymphomas in 21% nude mice, from the xenogenic cancer cells from 7 of the 8 individual oral cancer patients used. There is a paucity of information on lymphoma induction in nude mice by tumor xenografts. Lymphoma induction by different tumor xenografts has been reported to be as low as 1% (ref. 18), to as high as 58-63% (refs 20, 21). Moreover, absence of lymphomas in the control animals, indicates absence of development of spontaneous lymphomas as also a role for tumor xenografts/cells in development of the murine lymphomas. Several authors have reported a low level of 1-5% of spontaneous development of lymphomas in nude mice and at an average age of 9-24 months.

In the present studies, the absence of positive immunohistochemical staining of the lymphomas with human T cell, B cell and LCA antibodies indicate that the lymphomas are not of human origin. Studies by Wakasugi et al. have demonstrated murine lymphoma induction of TcR alpha/Beta(+) and CD4-8- phenotype following implantation of human inflammatory breast cells in nude mice. On the other hand, Baird et al., demonstrated induction of murine lymphomas which were a mixture of B, T and null cell population. In the present studies, using several mouse and human antibodies, 12% of the lymphomas were demonstrated to be of B phenotype. However, majority of the lymphomas failed to react with the murine T cell and B cell antibodies and it is likely that the lymphomas may be a null cell population.
The low level of specific lymphoma induction on xenogenic transplants reported in the nude mice suggests involvement of certain, as yet unidentified factors. The postulated mechanism of transformation of host cells may implicate activation of oncogenes\(^\text{24}\), presence of a host virus\(^\text{25}\), cell fusion\(^\text{26,27}\) or growth factors\(^\text{28-29}\). The possibility of tumor antigens on oral cancer tissues being immunosuppressive is an alternative. The presence of a protein designated immunosuppressive acidic protein (IAP) in the sera of oral cancer patients, and significantly differentially expressed in the control patients without oral cancer, has been reported\(^\text{30}\). Further, several studies on cell lines and/or transgenic mice demonstrate a role for bcl2 protooncogene in B cell longevity with consequent additional genetic events resulting in B-cell neoplasia\(^\text{31,32}\). Hence activation of bcl2 in the mouse lymphocytes, may provide the right milieu for generation of lymphomas. The mechanism of induction of these murine lymphomas by the inoculated human oral tumor cells, remains to be elucidated.

References


