Publications


4. **Hussain T**, Kotnis A, Sarin R and Mulherkar R. Genetic Susceptibility to Multiple Primary Neoplasms in the Upper Aero-Digestive Tract: Genotype Score and Phenotype Correlation. (communicated to *Cancer letters*)

Poster/oral presentations


2. 13th Human Genome Meeting “Genomics and the future of medicine”, India, 2008. (Poster presentation)

3. 29th Annual Convention of Indian Association for Cancer Research, India, 2010. (Oral presentation)

4. 6th Graduate Students Meet, ACTREC, Navi Mumbai, 2010. (Poster award)

5. 30th Annual Convention of Indian Association for Cancer Research and International Symposium on “Signalling Network and Cancer”, CSIR-IICB, Kolkata, 2011. (‘Rajnikant Baxi’ poster award)


Establishment & characterization of lymphoblastoid cell lines from patients with multiple primary neoplasms in the upper aero-digestive tract & healthy individuals

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Background & objectives: A major drawback for genetic studies as well as long-term genotype-phenotype correlation studies in cancer is lack of representative human cell lines providing a continuous source of basic biomolecules and a system to carry out various experimental investigations. This can be overcome to some extent by establishing lymphoblastoid cell lines (LCLs) by infecting peripheral blood lymphocytes with Epstein Barr virus (EBV) which is known to immortalize human resting B cells in vitro giving rise to actively proliferating B-lymphoblastoid cell lines. The present study involves preparation and characterization of LCLs generated from patients with multiple primary neoplasms (MPN) of upper aero-digestive tract (UADT).

Methods: Thirty seven LCLs were established from UADT MPN patients and healthy age, sex and habit matched controls using EBV crude stock. Characterization was done with respect to expression of CD-19 (Pan B-cell marker), CD3 (T cell specific marker), CD56 (NK-cell specific marker), cell morphology, ploidy analysis, genotype and gene expression comparison with the parent lymphocytes.

Results: LCLs showed rosette morphology with doubling time of approximately 24 h. Ploidy analysis showed diploid DNA content which was maintained for at least 30 population doublings. When compared with parent lymphocytes there appeared no change at genetic and gene expression level.

Interpretation & conclusions: Our results show that lymphoblastoid cell lines are a good surrogate of isolated lymphocytes bearing their close resemblance at genetic and phenotypic level to parent lymphocytes and are a valuable resource for understanding genotype-phenotype interactions.

Key words Epstein Barr virus - lymphoblastoid cell lines - multiple primary neoplasia - ploidy analysis - population doubling

Squamous cell carcinoma of the upper aero-digestive tract (UADT), comprising head, neck, oesophagus, trachea and lungs, are common cancers worldwide and one of the most common cancers found in Indian men1. Further, a 3-7 per cent annual risk of development of a second primary neoplasm among the survivors of early stage UADT cancer poses an additional threat in terms of morbidity and mortality2. Cumulative evidence from the case-control studies analyzing polymorphisms in xenobiotic metabolism,
DNA repair and other gatekeeper mechanisms suggest aberrant gene-environment interactions to be an important aetiologial factor in the genesis of multiple primary neoplasm (MPN)\textsuperscript{3,4}. Thus it is becoming increasingly important to validate the findings of huge number of genotyping studies in at least a subset of the patients by phenotypic assays. Short term studies to assay chromosomal aberrations, mutagen sensitivity assays and DNA repair kinetics have been carried out using lymphocytes or short term lymphocyte cultures derived from study population\textsuperscript{5-7}. However, limited availability of biological material and lack of reproducibility has been the major limitation of such studies.

Immortalization of human B lymphocytes by Epstein Barr virus (EBV) in vitro is used routinely to establish lymphoblastoid cell lines (LCLs). Infection by EBV transforms resting B cells from human peripheral blood into actively proliferating LCLs\textsuperscript{8}. Unlike SV40 or human papilloma virus (HPV), EBV allows cell immortalization with minimal genetic and phenotypic aberrations, with ease of establishment and maintenance making LCLs ideal material for genotypic and phenotypic studies\textsuperscript{8}. These provide an unlimited source of biomolecules like DNA, RNA or proteins and are a promising in vitro model system for genetic screening studies, genotype-phenotype correlation studies, a variety of molecular and functional assays along with immunology and cellular biology studies\textsuperscript{9-12}. Utility of LCLs is also been very well documented in various population based studies especially investigating in vitro carcinogen sensitivity and DNA damage/repair\textsuperscript{13-15}. Here we report generation of 37 LCLs from patients with UADT MPN and healthy individuals and characterization of a few randomly selected cell lines.

### Material & Methods

This study was approved by the Hospital Ethics Committee, Tata Memorial Centre, Mumbai and conducted during 2005-2009 in the Mulherkar laboratory of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, Maharashtra, India. Samples from healthy, age, sex and habit matched, control individuals were obtained from staff and students of ACTREC, Navi Mumbai.

**Collection of samples:** After obtaining IRB approval and patient informed consent, 3 ml whole blood was collected in an EDTA vacutainer from patients with MPN and cancer free healthy individuals by venipuncture. Patients with MPN were accrued from the Cancer Genetics Clinic in Tata Memorial Hospital and samples from healthy control individuals were obtained from Advanced Center for Treatment Research and Education in Cancer, Mumbai, India. The study was approved by the Hospital Ethics Committee, Tata Memorial Hospital, Mumbai. UADT MPN patients were recruited on the basis of criteria given by Hong et al\textsuperscript{16} as described earlier\textsuperscript{17}.

**Generation of viable EBV stock:** EBV-transformed B95-8 marmoset cell line was procured from National Centre for Cell Sciences, India and the EBV stock prepared. Briefly, 0.5x10\textsuperscript{6} cells/ml were seeded in RPMI-1640, 15 per cent foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 200 mM glutamine (Sigma-Aldrich Co., USA) and 1X PenStrep (Sigma-Aldrich Co., USA). After 7 days confluent cultures of B95-8 appearing straw yellow in colour were lysed by freeze thawing at -80°C and 37°C and filtered through 0.22 μm filter (Millipore, Bedford, MA) to obtain EBV crude stock. The filtrate was aliquoted and stored at 4°C for short term or -80°C for long-term storage.

**Lymphoblastoid cell line preparation:** For separation of peripheral blood lymphocytes (PBLs) approximately 3 ml of blood was separated on a Ficoll-Hypaque gradient (Sigma-Aldrich Co., USA). PBLs were seeded in a sterile 24 well plate at a density of 1.5-2x10\textsuperscript{6} cells/m1 in Dulbecco’s minimal essential medium (DMEM) containing 15 per cent FBS 200 mM glutamine and 1X Pen Strep. EBV crude stock at 1:1 ratio was added and placed in an incubator maintained at 37°C with 5 per cent CO\textsubscript{2}. After 24 h medium containing viral supernatant was aspirated without disturbing the cells and fresh complete DMEM was added. After 3-4 wk of incubation rosette morphology of cells ascertained the transformed phenotype of PBLs. Cells were mixed thoroughly to break clumps before splitting to ensure monoclonal population. All the used plasticware (Nunc, Denmark) was treated as biohazard, discarded in 1 per cent sodium hypochlorite after use and autoclaved wherever required.

**Characterization of LCL:** Characterization of LCLs was done using standard techniques as described below. For all assays freshly grown cells with >90 per cent viability were harvested and washed in 1X PBS at 1500 rpm and used for the genotypic and phenotypic characterization.

**Immunophenotyping:** 1x10\textsuperscript{6} cells from LCLs were incubated with primary mouse anti-CD3 (T cell
marker), anti-CD19 (pan-B cell marker) and anti-
CD56 (NK cell marker) antibodies (BD Pharmingen,
BD Biosciences, USA) for 45 min on ice. The cells
were washed thrice with FACS buffer (PBS containing
1% FBS and 0.02% Na-azide) and incubated for 45
min on ice with secondary goat anti-mouse FITC
antibody (antibodies used in this experiment were kind
gift from Dr Shubhada Chiplunkar and Dr Naren Joshi,
Immunology department, ACTREC). Cells were passed
through BD™ 1ml 26G ½ syringe (BD, Singapore)
to break any cell aggregates or clumps and analyzed
on Flow Cytometer (FACS Calibur, BD Biosciences,
USA) at 488 nm excitation. A minimum of 10,000
events were analyzed for each sample. Cellular debris
was removed by gating on Forward vs. Side Scatter.
Statistical analysis was done using CELLQUEST
software (BD Biosciences, USA).

Ploidy analysis of LCLs: LCLs (1x10⁶) and control
PBLs (1x10⁶) from healthy volunteer were fixed in 70
per cent ethanol at 4°C for 1 h. The cells were washed
twice in PBS followed by incubation with propidium
iodide (Sigma-Aldrich Co., USA) and RNaseA (Sigma-
Aldrich Co., USA) for 30 min at 37°C. Fluorescence
was acquired on Flow Cytometer at 488 nm excitation.
Cells were passed through BD™ 1ml 26G ½ syringe
before acquisition to break any cell aggregates or
clumps and a minimum of 10,000 events were analyzed
for each sample. Data were analyzed using ModFit LT
V 2.0 software (BD Biosciences, USA). DNA ploidy
is defined as diploid DNA represented as single G0/
G1 peak on a histogram corresponding to the same
DNA content represented as single G0/G1 at the same
position in the histogram of the control. Ploidy was
measured by calculating DNA index (DI) which is the
ratio between the channel number of G0/G1 peak on
histogram of the cell line to the channel number of G0/
G1 peak of control PBLs.

Expression of ATM gene: RNA was isolated from cell
lines and lymphocytes isolated from the same subjects
by TRIzol (Invitrogen, Carlsbad, CA) extraction
method. β actin PCR¹⁸ was performed on isolated RNA
to ensure any DNA contamination and samples were
治 treated with DNase using DNA-free kit (Ambion,
Austin, TX) wherever required. cDNA was synthesized
using 3 μg of total RNA using Superscript First-Strand
synthesis system by RT-PCR (Invitrogen, Carlsbad, CA)
according to manufacturer’s instructions. Expression
of ATM gene was measured semi-quantitatively by RT-
PCR using gene specific primers (Sigma-Aldrich Co.,
India; Forward 5'-TGTCATTACGTAGCTTTC-3';
Reverse 5’-GCTGAGTAATACGC AAATCC-3) and β
actin was used as loading control. PCR products were
run on 2 per cent agarose gel and stained with ethidium
bromide.

Cell population doubling: 5x10⁴ cells from LCLs were
seeded in a 24 well plate with 1.5 ml of complete
medium. Viable cell count was taken using Trypan
blue dye exclusion method¹⁹ at different time points
including 0, 12, 24, 36, 48, 72 and 96 h. For each time
point four readings were taken.

Results

Establishment of LCLs and morphological analysis:
Blood samples from MPN patients (n=24) and cancer
free control individuals (n=13) were obtained and
subjected to LCL preparation. The demographics of
the patients and control individuals are shown in Table
I. Considerable cell death of the PBLs was observed
after 24 h post EBV infection; however, virus infection
promoted B cells to re-populate the culture. The time
taken for each LCL preparation varied. On an average
culturing the cells 3-4 wk post infection was sufficient
to produce >1 million cells.

The average population doubling (PD) time of a
few representative LCLs was found to be 24 h, ranging
from 12 to 36 h (Fig. 1). The LCLs grew as clusters
exhibiting typical rosette morphology in suspension
cultures, but single cells were also observed having
big nucleus and numerous vacuoles (Fig. 2a, 2b). EBV
is known to specifically infect B cells allowing their
growth in culture hence the transformed phenotype is
expected to have homogeneous cell population, however,
flow cytometric analysis revealed the presence of dual
population (Fig. 2c). On the basis of morphology and
granularity it was revealed that lower (R1) population
represents mono cell suspension of interest and the
other (R2) population probably represents fraction of
cells that have spontaneously differentiated into smaller
lymphoid cells with shrunken nucleus that ultimately
undergo apoptosis during conventional cell culture
thus representing diverse size and granularity²⁰.

Cell surface marker: Immunophenotyping was done
using PBL as positive control for B (CD19), T (CD3)
and NK (CD56) cells. Data confirmed that cells from
representative randomly selected LCLs showed
expression of typical B cell surface marker (CD19)
while markers for T cell (CD3) and NK cells (CD56)
were absent (Fig. 3), thus ascertaining the purity of
growing cultures.
<table>
<thead>
<tr>
<th>No.</th>
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<th>2nd Cancer</th>
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<td>M</td>
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<td>Larynx</td>
<td>Hard palate</td>
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<td>E311</td>
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<td>Left lower lip+ right lower lip+ mouth</td>
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<td>MPN</td>
<td>GBS</td>
<td>GBS+ RMT+ alveolous</td>
</tr>
</tbody>
</table>

MPN, multiple primary neoplasm; GBS, gingivobuccal sulcus; AML, acute myeloid leukaemia; RMT, retromolar trigone; M, Male; F, female
DNA ploidy analysis: DNA ploidy status of the LCLs was assessed immediately after cell line preparation at low population doubling (<5 PD) using PBL from healthy individuals as diploid control. A shift in the position of the diploid peak of LCL away from the expected diploid position of the control can be taken as an evidence of DNA aneuploidy. All the cell lines studied had DI values ranging between 0.8-1.3 and were considered diploid (Table II, Fig. 4). Cell lines in continuous culture can show aberrant DNA content hence ploidy was measured even at higher population doublings of 30, 45, 60, 120 and 150 in a few cell lines. The DI values ranged between 0.93 and 1.18; hence were considered to be diploid (Table III).

Expression and activity of ATM gene: Lytic cycle of EBV elicits a cellular DNA damage response resulting in activation of the ataxia telangiectasia-mutated (ATM) signal transduction pathway\textsuperscript{21}; hence ATM expression in the cell lines and their respective PBL was studied. There was apparently no change in the expression level of ATM gene between the LCL and respective PBL from the subject (Fig. 5). The number of pATM Ser1981 foci seen as distinct nuclear foci in response to cellular DNA damage, were similar in both - the cell line as well as lymphocytes (data not shown).

Discussion

We report here generation and characterization of LCLs from MPN patients and healthy individuals. LCLs can be generated efficiently as continuously growing cells from an individual following infection of PBLs with EBV-containing supernatants. Such EBV immortalized cells are derived from B lymphocytes and, unlike cell lines derived from other tissues, remain near diploid in nature. Such LCLs can be used for various \textit{in vitro} studies as well as serve as a source of DNA for genomic studies. Generation of EBV transformed cell lines has proven to be cost-effective, rapid and reliable with minimal deviation from the normal phenotype and genotype.

B95-8 cells derived from Marmoset lymphocytes, infected and immortalized with EBV, are typically used for virus production since these spontaneously

**Fig. 1.** Graph showing cell population doubling time for 6 representative cell lines at 6 time points till 96 h. The doubling time ranged from 12 to 36 h with an average of approximately 24 h. Values are ± mean SEM (n=4).

**Fig. 2.** Morphological analysis of established lymphoblastoid cell lines (a) Light microscopy image of LCL showing typical rosette morphology. Cells grow in clumps while single cells are also seen. (b) Confocal microscopy image of cells in EBV transformed cell lines showing big nucleus and numerous vacuoles. (c) Flow cytometry cluster plot showing two distinct population in LCL where lower R1 population represents mono cell suspension and R2 population represents cell aggregates.
Fig. 3. Flow cytometry analysis for cell surface markers - CD3 (T cell), CD19 (B cell) and CD56 (NK cell) in representative cell lines. Unstained cells were used as internal control. X-axis represents the fluorescence intensity. A forward shift in the peak, caused by binding of fluorophore tagged antibody, as compared to unstained cell is considered positive (a) LCLs E242, E265, E245 and E247 were positive for CD19 and negative for CD56 marker while (b) LCLs E252, E253, E246 and E249 were positive for CD19 and negative for CD3 marker.
produce the B95-8 strain of EBV\(^{22}\). Average time taken for LCL establishment was 3-4 wk extending up to 5 wk in some cases. Variation in the time taken for cell line establishment and growth rate can be attributed to difference in the transformation efficiency occurring due to possible batch-wise difference in the viral titre. Once the cell lines were established, these behaved similarly showing comparable morphology, doubling time, genotypic and cell surface characters and behaviour in phenotypic assays.

Successful transformation of B cells by EBV resulted in enlargement in size and development of aggregates of proliferative cells. Due to the acquired property of cell aggregation LCLs grew as clumps in suspension cultures, with a mean population doubling time of 24 h and when seeded at a density of 0.5-1X10\(^6\)/ml needed to be split twice every week. Early passage cells were cryopreserved immediately after transformation. Established LCLs were cryopreserved at later passages as well, but not later than 30 population doublings.

EBV transformed LCLs are known to exist in two distinguishable forms pre-immortal and post-immortal. In the pre-immortal stage cells proliferate actively and maintain diploid karyotype. These cells are non-tumorigenic and die before reaching 160 population doublings. On the other hand, in the post immortalization stage, EBV transformed cells develop a strong telomerase activity and are aneuploid. These also show cellular changes, gene mutations and have the ability to grow indefinitely\(^9\). Hence, in the present study, for phenotypic assays LCLs were used within 45-60 PD.

Morphological analysis of cell lines by flow cytometry showed two distinct populations although as EBV is known to specifically transform B cell, only one population is expected. The R1 population represents B cells, based on cell morphology and granularity, and R2 population could be proliferating T/NK cells due to immune response elicited by EBV infected B cells. Conventionally the immune suppression of T cells in LCLs is done by supplementing LCL cultures with cyclosporine-A post infection to improve immortalization\(^8\). However, no cyclosporine A was added in the present study. To study the nature of cells in both clusters immunophenotyping was done using CD19, CD3 and CD56 antibodies in a few representative cell lines. All the cell lines tested were positive for B cell marker in both the clusters (data not shown). The possible reason for occurrence of R2 population could be differential size and granularity of the cells arising due to spontaneous differentiation into smaller lymphoid form with shrunken nucleus. These cells in usual cell culture further undergo apoptosis\(^{20}\).

### Table II. DNA ploidy status of MPN patient and healthy control cell lines

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<thead>
<tr>
<th>LCLs</th>
<th>Channel ratio (LCL/PBL)</th>
<th>DNA index</th>
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<tbody>
<tr>
<td>E245</td>
<td>63.75/49.62</td>
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<td>E246</td>
<td>53.83/49.62</td>
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<td>57.64/49.62</td>
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<tr>
<td>E306</td>
<td>49.37/46.58</td>
<td>1.05</td>
</tr>
<tr>
<td>E307</td>
<td>49.61/46.58</td>
<td>1.06</td>
</tr>
</tbody>
</table>

PBLs from healthy individual were used as reference. Values in the range 0.9-1.3 are considered diploid.
Cell immortalization and proliferation in continuous cultures can result in aberrant DNA changes like aneuploidy or tetraploidy. Hence ploidy status of the cell lines was ascertained at both, low and high population doublings ranging from <5 to >100 PD taking normal PBL as control. DNA index (DI) ratio was calculated to establish the ploidy status. DI ratio of 1 was considered as diploid while DI values ranging from 1.9-2.1 with proportion of cells greater than the G2/M fraction of normal control, after correction of the aggregates were considered as tetraploid. All the cell lines studied at lower (<5 PD) as well as higher population doubling (>100 PD) had a DI ratio ranging between 0.9-1.3 and were considered to be diploid.

As described in earlier reports EBV remains episomal in lymphoblastoid cell lines maintaining a latent infection, although there is a small subpopulation of cells that switches spontaneously from a latent stage of infection into the lytic cycle. Induction of EBV lytic replication elicits cellular DNA damage response dependent on $ATM$. DNA damage sensor MRN (Mre II, Rad 50 and Nbsl) complex and phosphorylated $ATM$ are recruited to viral replication compartments, presumably recognizing newly synthesized viral DNAs as abnormal DNA structures. The LCLs in the present study were established with an aim to eventually study the contribution of DNA damage repair in vitro in UADT MPN patients and to elucidate the mechanism involved. Hence it was necessary to ensure that the process of EBV transformation did not affect expression and activity of DNA repair gene $ATM$. It was observed that EBV transformation did not elicit DNA repair pathway dependent on $ATM$ as there were no pATM foci seen in LCLs (data not shown). This was further confirmed by measuring $\gamma$H2AX foci in cell lines which was also found to be negative (data not shown). Also there was no change in $ATM$ gene expression in cell lines and PBLs as revealed by semi-quantitative RT PCR data.

This property of LCLs to be able to grow in continuous culture together with maintaining a close similarity to the parent lymphocytes has been exploited in various studies. There are numerous reports where LCLs have been used as a source of basic biomolecules.

**Fig. 4.** Flow cytometry analysis showing the diploid status of PBLs isolated from healthy subject and two representative LCLs E306 and E307. The black arrows in the histogram X- axis represent the channel number of the respective cell cycle stage. Arrow at smaller channel number corresponds to G0/G1 stage of cell cycle while the other arrow corresponds to G2/M stage which is located at approximately double position.

**Fig. 5.** Expression of $ATM$ gene in different cell lines and their respective PBLs (a) E302 (b) E303 (c) E313. RT-PCR products were run in 2 per cent agarose gel stained with ethidium bromide. $\beta$-actin gene was taken as loading control.
like, DNA, including mitochondrial DNA, RNA and protein. DNA isolated from LCLs has been widely used for mutation analysis, while RNA isolated from these cell lines has been commonly used for cDNA library preparation and to assess transcriptional response to genotoxins using high throughput technologies including cDNA microarray, together with this LCLs have as well been used for proteomic studies.

For large scale population based studies, LCLs provide a constant supply of starting material for a variety of assays, sparing the need of re-sampling. LCLs have been established as an excellent model system not only in basic biomedical studies but also to carry out genomic wide high throughput research thus showing their utility in a broad range of biomedical research. All this emphasizes the research utility of LCLs as a surrogate for isolated lymphocytes. The cell lines developed in the present study have been well characterized and provide a valuable, cost effective, in vitro model system for genotypic and phenotypic assays ensuring adequate starting material for current and future analysis.

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CASE REPORT
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SQUAMOUS CELL CARCINOMA OF BASE OF TONGUE IN A PATIENT WITH FANCONI’S ANEMIA TREATED WITH RADIATION THERAPY: CASE REPORT AND REVIEW OF LITERATURE

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Abstract: Background. Fanconi’s anemia (FA) is a rare autosomal recessive genetic disorder characterized by congenital anomalies, progressive aplastic anemia, and a predisposition for malignancies.1 Congenital anomalies in FA clinically manifest as skeletal, renal, ophthalmological malformations, and chromosomal aberrations. The disease involves multiple organs that include skin, genitourinary, musculoskeletal, renal, and neurological systems. The clinical findings in FA patients are abnormal skin pigmentation like cafe au lait spots, abnormal male gonads (absent, atrophic, or abnormal

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testis, hypospadias, undescended testis), microcephaly, short stature, hypoplastic thumb with or without radial anomalies, renal defects, and developmental delay, mental retardation, and learning disability. FA is associated with increased risk of malignancies, and the risk further increases with bone marrow transplantation, which is done for treatment of hemopoietic failure associated with the disease.

The cause of development of cancer in FA is thought to be attributable to a defect in maintaining the genome integrity, leading to increased chromosomal instability with defective DNA repair mechanism. Although hematological malignancies are 1 of the most common cancers, solid tumors in the head and neck region, especially in the tongue, are also observed. Management of cancers in the head and neck region in patients with FA is challenging. Surgery, although considered as an optimal treatment, has its limitations as a result of low counts, risk of infection, and wound-healing issues.

Radiation therapy or chemotherapy is associated with increased risk of toxicity in these patients because of defective DNA repair mechanism. There have been instances of variable clinical radiosensitivity and fulminant radiation toxicities even with low doses of radiation. Therefore, in vitro sensitivity studies, although not common, have been performed on fibroblast cells or peripheral blood mononuclear cells to predict the in vivo radiation sensitivity. We report a case of a young man with FA who was diagnosed with carcinoma of the base of tongue and was treated with radical radiation therapy. We also report the results of an in vitro test for radiosensitivity in our patient and review the literature for FA patients with head and neck cancer.

**CASE REPORT**

A 27-year-old man was referred to our hospital for evaluation of a base-of-tongue lesion that had appeared 3 months earlier. Oral examination revealed a well-circumscribed, ulceroproliferative lesion on the left side of the base of tongue measuring 2.5 cm in diameter. Neck examination showed no cervical lymphadenopathy. On general physical examination, he was physically retarded (short stature, microcephaly, and microophthalmia) with atrophic testis and a few café au lait spots on skin, particularly of the lower extremities.

MRI showed a well-localized soft tissue mass on the base of tongue (left > right), with no significant cervical lymphadenopathy (Figures 1A and 1B). The histopathology review showed squamous cell carcinoma. Therefore, a diagnosis of carcinoma of the base of tongue, T2N0M0 (stage II), was made. The complete blood count, showed features of generalized pancytopenia (total leukocyte count, 1.8 × 10^9/L; hemoglobin, 7.1 gm/dL; and platelets, 17 × 10^9). Complete blood count was repeated but pancytopenia persisted. The patient underwent a bone marrow aspiration and biopsy, which revealed hypocellular marrow. In view of his abnormal morphological features and unexplained hypocellular marrow, the patient was advised for chromosomal studies. Subsequently, chromosomal breakage analysis was done in peripheral blood lymphocytes in the presence of DNA crosslinking agents, diepoxybutane (DEB) and mitomycin C (MMC), which revealed a high frequency of chromosomal breakage (6.2×10⁻³).
chromosome breaks per metaphase) compared with control (1.3 breaks per metaphase). Increased frequency of radial formation was also observed in this patient compared with controls (Figure 2). Bone marrow chromosomal analysis further revealed chromosomal aberrations. Finally, diagnosis of FA was made on the basis of clinical features, hematological picture, and chromosomal breakage analysis.

Hematological parameters precluded surgical interventions and chemotherapy. It was therefore decided to treat him with radical external beam radiation therapy. Radiation to the face and regional lymph nodes was started with conventional fractionation of 200 cGy/day once daily and 5 days/week schedule using bilateral parallel opposed portals. In week 4 of radiation, a nasogastric tube was inserted to improve the nutritional support. The patient was admitted after completion of 54 Gy, given that his total leukocyte count was $2.1 \times 10^9/L$ and he developed fever. Broad-spectrum injectable antibiotics were started, along with granulocyte colony stimulating factor. He successfully completed the treatment to a total dose of 70 Gy/35 fractions over 51 days with reducing portals respecting the tolerances of critical structures. The treatment was completed without any interruptions. At the conclusion of treatment, he had Radiation Therapy Oncology Group (RTOG) grade 3 toxicity of skin and mucosa. His count was consistently low, for which he was kept on intensive supportive care. Admission was required because of systemic effects, and not because of radiation reaction. He was eventually discharged from the hospital after 4 weeks, when his fever had subsided but pancytopenia persisted.

In vitro study was done to determine the radiosensitivity by radiating peripheral blood mononuclear cells (PBMCs) isolated from blood to increasing doses of radiation. The rate of apoptosis in this patient was compared with a matched control. At each radiation dose as well as in the unirradiated sample, FA had more live cells than that of control. Spontaneous rate of apoptosis (ie, apoptosis at 0 Gy) was found to be less in this case of FA. The percentage of early apoptotic cells increased from 9% to 30% in the case of an FA patient with an increase in radiation dose from 0 Gy to 25 Gy compared with control, in which it increased from 14% to 48%. The percentage of late apoptotic cells was also found to be low in the case of the FA patient, which increased from 2% to only 5%, which in the case of control reached 14%. This experiment suggested that radiation-induced apoptosis was less in our patient. We also studied the

FIGURE 2. Chromosomal breakage analysis in patient with Fanconi's anemia shows high frequency of chromosomal breaks.
percentage of necrotic cells and observed that the percentage of necrotic cells was greater in the case of the FA patient compared with control. Necrotic cells increased from 1.5% to 12% in the case of the FA patient, compared with control, in which they remained <2% at each radiation dose, suggestive of cell death attributed to the necrotic inflammatory pathway in the FA patient, making them more radiosensitive.

The patient was followed up at 6 weeks post-radiation therapy, when he was evaluated for local control and toxicities. The radiation reactions of skin and mucosa had settled and there was no clinical evidence of disease. A follow-up evaluation at 3 months postradiation therapy revealed similar findings. An MRI scan showed evidence of level IV lymph node. This lymph node was not present in the preradiation therapy scan. Fine-needle aspiration cytology from this node showed the presence of squamous carcinoma. Opinion was taken for surgical salvage but was not considered because of fixation to surrounding structures, low counts, and the risk associated with surgery because of his FA. He was therefore considered for palliative reirradiation with electrons, which he completed with good symptom relief. He was alive with disease at his last follow-up, which was 14 months after his initial diagnosis.

**DISCUSSION**

FA is an autosomal recessive disorder associated with bone marrow suppression, congenital anomalies, and high risk of malignancies.17 It has been hypothesized that the malignancies occur either because of chromosomal instability or because of immunodeficiencies.18 Although the association of FA with malignancies was described in 1927, cancer of the head and neck region was reported in 1966 by Esparza and Thompson.9 Hematological malignancies are the most common malignancies seen in patients with FA, followed by solid tumors, especially head and neck cancers.9 In a large study of 1300 patients with FA, the incidence of solid tumors was around 5%.19 In a review of 754 patients from the International Fanconi’s Anemia Registry, 3% patients had head and neck cancer.20 This incidence was significantly higher compared with that of the normal population.9 Although the data on ethnic origin of these patients are not very clear, there is a scarcity of data from Asian countries. This is thus 1 of the important case reports in an Indian male patient.

The median age of onset of tumors in patients with FA is as early as 16 to 31 years in different series.12,19 In our patient, the age of onset was at 27 years, which was much earlier compared with the median age of 56 years in our population for head and neck cancer. In our patient, the diagnosis of malignancy was made before that of FA. His routine investigations showed pancytopenia, which led to further investigations, such as bone marrow biopsy and chromosomal studies, which eventually confirmed the diagnosis of FA. Surgery has been considered as the primary modality of treatment in many patients with FA.12 Of the 19 patients with head and neck cancer in the International FA Registry, 17 have undergone primary surgery. Surgery in these patients was well tolerated.9 It has been suggested that surgery should be encouraged in these patients, to prevent the issues associated with chemotherapy and irradiation.9,13,14,20

Chemotherapy—associated with very high morbidity attributed to DNA damage and impaired repair mechanism—was thus not considered in our patient. In addition, this patient had stage II low-volume disease; therefore single-modality treatment in the form of radiation therapy was considered. Use of alkylating agents that crosslink DNA can have serious adverse effects in these patients.14 Furthermore, chemotherapy is also known to cause a deleterious effect of myelosupression in patients with baseline bone marrow suppression.9,12–14

There have been varying reports of tolerance to radiation therapy in cancer patients with FA and there appears to be increased radiosensitivity in these patients.13,14 This could possibly be ascribed to an increase in chromosomal breakage and impaired cell repair mechanisms.9,13,14,16 In 1 of the reported cases, the radiation mucositis was observed as early as 3.2 Gy, whereas in another patient radiation was delivered without much toxicity.12 Marcou et al15 reported a patient with tonsillar cancer who developed unusually brisk reactions after 24 Gy. Varying doses of radiation have been documented in the literature, ranging from 3.2 Gy to 80 Gy. Overall, radiation has been associated with increased normal tissue toxicity, delayed healing, and increased supportive care.
FA along with ataxia telangiectasia, Bloom's syndrome, and xeroderma pigmentosum are called cancer breakage syndromes because they are associated with chromosomal instability and defective repair, thereby predisposing to development of cancers.  

Cells from FA show variable levels of cellular radiosensitivity, and results of an in vitro experimental study done for predicting the hyper-sensitive response may not coincide with in vivo radiosensitivity results. Various experimental techniques including alkaline single-cell gel electrophoresis (Comet assay), colony-forming test, Western blot, and foci immunofluorescence analysis of the expression of DNA repair proteins, and also the cytochalasin-blocked micronuclei (MN) test using FA fibroblasts have been adopted to correlate the in vitro radiation sensitivity with the outcome of therapy. However, the limitation of these techniques is the 4-week delay required for colony growth. Comet assay is widely regarded as a robust and informative method for radiosensitivity measurements immediately after DNA damage, but it is only a qualitative assessment of the response. Western blot and foci immunofluorescence analysis give qualitative results, but are labor intensive and also lack correlation between in vitro results and in vivo radiation response. In view of the conflicting data on radiosensitivity of FA cells, and particularly FA fibroblasts, there is a pressing need for the development of new rapid and predictive assays of radiation responses. We have performed our study on PBMCs isolated from blood. Compared with most of the previous studies using fibroblasts, collecting blood has an advantage over invasive procedures of obtaining fibroblasts from patients' skin biopsies, avoids time-consuming methods of generating monolayer cultures, and is also less labor intensive.

Clinical radioresponsiveness is unpredictable in patients of FA with cancer, especially of the head and neck region, and outcome varies. Of the 19 patients in the International FA Registry who had head and neck cancer, 14 died because of disease. The median time to recurrence in their patients was 16 months and the median follow-up of surviving patients was 19 months. Kennedy and Hart noted multiple primary malignancies either synchronous or metachronous in patients with FA to the extent of 36% (5 of 14 patients). Our patient developed a lymph node at level IV at 3 months follow-up and was alive with disease at 14 months.

There are no standard guidelines for management of FA patients with malignancies. This is primarily a result of the scarcity of data and heterogeneous population. It has therefore been recommended that more data in the form of case reports should be encouraged. Until then, the treatment of patients with malignancies with FA should be individualized. Any decision about opting for different modalities should be based on a balanced approach with respect to locoregional control and toxicities of the treatment.

REFERENCES