Chapter-3: Materials and Methods

3.1 Materials

3.1.1 ESCC Patient Selection:

From 2010-2015, 108 patients, who presented with locally advanced esophageal squamous cell carcinoma (ESCC) and treated with curative intent, were selected for this study. They received standard platin, taxan and 5 fluro uracil based chemotherapy regime as per national comprehensive cancer network (NCCN) guidelines. Endoscopies, Computed Tomography and X- ray were done to assess the disease status.

Endoscopic biopsies were taken for histopathology reporting and subsequent immunohistochemistry (IHC) tests. Another section of that biopsy was stored in RNAlater (a preservative media) at -20 degree Celsius for subsequent cell cycle analysis.

3.1.2 Ethical approval and consideration:

This study was approved by the Institutional Review Board of the Cachar Cancer Hospital & Research Centre, Silchar, India and tissue specimens along with clinical information were used as per their recommendations. The approval number of this study was (IRB/CCHRC/07/2013).

All the patients recruited in this study were from the Cachar Cancer Hospital & Research Centre, Silchar, Assam, India. Patient's identity on the clinical data was masked. All the chemicals and reagents used in this study were of molecular and analytical grade and were purchased from the reputed international brands.

3.1.3 Inclusion and Exclusion criteria:

(I) Inclusion criteria for the patient recruitment:

1. Informed consent.
2. Histo-pathologically proven esophageal squamous cell carcinoma.
3. Treated with curative intent.
4. Underwent neo adjuvant chemotherapy (NACT).
5. Availability of the post NACT response assessment data.
6. Age > 30 years.
7. *De novo* patient's i.e. those who received their cancer directed treatment for the very first time at this hospital.

(II) Exclusion criteria for the patient recruitment:

1. Histopathology reporting if not done or missing.
2. Upfront surgery if done.
3. Non availability of the post NACT treatment response assessment data.
4. Advanced esophageal untreatable cancer cases.
5. Age ≤ 30 years.
6. If the patient was treated elsewhere other than this hospital.

3.1.4 Chemicals and reagents:

(i) 3-Aminopropyltriethoxysilane (Sigma-Aldrich)

(ii) Hydrogen peroxide (Dako)

(iii) Xylene (Merck)

(iv) Propanol (Merck)

(v) Tris base (Sigma-Aldrich)

(vi) Phosphate buffered saline (PBS) (Sigma-Aldrich)

(vii) Ethylene diamine tetra acetic acid (EDTA) (Sigma-Aldrich)

(viii) Paraffin (Merck)

(ix) 3,3-diaminobenzidine (DAB) (Dako)

(x) Dibutylphthalate Polystyrene Xylene (DPX) (Merck)

(xi) Haematoxylin (Merck)

(xii) Eosin (Merck)

(xiii) Monoclonal antibody: Aldehyde dehydrogenase 1 (ALDH1) (Becton Dickinson)

(xiv) Polyclonal antibody: Human epidermal growth factor receptor 2 (HER2) (Dako)

(xv) Monoclonal antibody: $P16^{INK4A}$ (Santa Cruz Biotechnologies)

(xvi) Envision Flex secondary antibody detection system (Dako)

(xvii) Propidium iodide (PI) (Sigma Aldrich)

(xviii) Sodium citrate tribasic dihydrate (Sigma Aldrich)

3.2 Methods

3.2.1 Clinical, epidemiological and lifestyle information documentation:

Clinical data was extracted from the respective patient's medical record. Epidemiological and life style data like demography, age, gender, religion, smoking, tobacco chewing, alcohol use, areca nut and betel quid (pan) use were extracted from the patient medical record. Initial performance status (PS) of patient on their first visit was assessed by Eastern Cooperative Oncology Group (ECOG) scale as described in Table 4 (Oken, M.M *et al.* 1982).

Grade	ECOG performance status (PS)
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self care but unable to carry out any work activities; up and about more than 50% of waking hours
3	Capable of only limited self care; confined to bed or chair more than 50% of waking hours
4	Completely disabled; cannot carry on any self care; totally confined to bed or chair

Table 4. Description of the scoring system in ECOG performance status. Source: (Oken, M.M et al. 1982).

Data related to the patient's initial symptom, symptom duration, response to chemotherapy, biochemical parameter, pathological grading, disease site, circumferential disease location, and family history of cancer was extracted from the case record. Overall

survival was assessed from the month of diagnosis of ESCC to the death or the last follow up.

3.2.2 Treatment response assessment:

Neo adjuvant chemotherapy (NACT) (platin, taxan and 5 fluro uracil based chemotherapy regime) as per National Cancer Care Network (NCCN) guidelines was administered in all the studied ESCC patients with curative intent. NACT induced response assessment was performed by considering the status of tumor and its pathological/endoscopy/imaging report after completion of chemotherapy. Those patients who achieved complete remission i.e. without any pathological trace of tumor following chemotherapy were considered responders whereas those with any tumor residue or patchy disease were categorized as non responders in this study.

3.2.3 Immunohistochemistry of molecular markers (ALDH1, HER2, p16):

3-Aminopropyltriethoxysilane (Sigma-Aldrich) treated glass slide was used for adhering 4 μm tissue cut section from paraffin blocks. It was left for drying under fan for 1 hour and subsequently inside incubator at 50°C for 12 hour. Positive controls (liver tissue) and negative control (without primary antibody) were used in each batch of experimentation. Slides were heated at 60°C for 1 hour that was followed immediately with 2 step xylene (Merck) wash, 10 minutes each for deparaffinization. Thereafter, it was rehydrated with graded alcohol for a total of 20 minutes and was kept for 1 minute in distilled water.

Antigen retrieval was performed by placing slides in pre-warned (65°C) Triss-EDTA (pH 9; Dako) buffer and heating it at 97°C for 20 minutes in microwave oven. After cooling it to room temperature, blockage of endogenous peroxidase was performed by immersion in

3% hydrogen peroxide (Dako) for 10 minutes. After 5 minutes wash with phosphate buffered saline (PBS), sections were incubated with 3 primary antibody on 3 separate tissue slide: anti-human ALDH1 (Clone 44/ALDH; 1:200; Becton Dickinson, USA), anti-human P16^{INK4A} (Clone JC8; dilution 1:150; Santa Cruz Biotechnology, USA) and anti-human HER2 polyclonal antibody (Clone A0485, 1:400; DAKO Agilent, Denmark) in humidified chamber for 1 hour. Excess primary antibody was washed with PBS. Envision Flex/HRP (Dako) was applied as secondary antibody over slides and incubated for 30 minutes in humidified chamber. After washing slides with PBS, 3,3-diaminobenzidine (Dako) was applied as chromogen for 10 minutes. Lastly, sections were washed with distilled water, counterstained with haematoxylin for 30 seconds, rehydrated with graded alcohol 2 minutes each, dried and mounted with DPX for examination under microscope. Additionally, haematoxylin and eosin staining was done for each sample to confirm and identify the cancer and normal epithelial cells in ESCC (Sunpaweravong, Sunpaweravong et al. 2016). Figure 10 depicts the basic steps of immunohistochemistry.

Figure 10. Schematic representation of a basic immunohistochemistry experiment.

3.2.4 Immunohistochemical scoring of molecular markers (ALDH1, HER2, p16):

ALDH1 and HER2 IHC scoring was done by 2 independent pathologist, blinded to this study. Scoring system for ALDH1 reported by Bin Chang was used. Briefly, section's with < 5% ALDH1 positive cells were assigned 0 score, those with 5 to 20% ALDH1 positive cells were assigned a score of 1, those with 20-50% ALDH1 positive cells were given a score of 2 and those having more than 50% of ALDH1 positive cells were assigned a score of 3 (Bin Chang et al, 2009). For the purpose of statistical analysis ALDH1 reactivity in greater than 10% of tumor cells were considered positive.

Bang YG recommended scoring system for HER2 was utilised. Score 0 was assigned in those sections where no HER2 positive cells were seen, score 1 was assigned in those where a faint or barely perceptible membrane staining of HER2 in cancer cells was seen, score 2 was assigned in those where a weak to moderate bilateral or lateral HER2 staining in cancer cell was visible and score 3 was assigned in those sections where a strong complete, basolateral, or lateral staining of HER2 in 5 or more cluster of cancer cells was seen (Bang Y-J et al, 2010). During analysis, HER2 score 0,1 and 2 were assigned negative and HER2 score 3 was assigned positive.

Strong and uniform cytoplasmic and nuclear p16 staining in all or most cancer cells were considered p16 positive on the contrary weak or absent p16 staining on tumor section were considered as negative (El-Naggar and Westra 2012). Tumor sections that showed strong p16 expression in more than 50% for tumor cells was considered as positive and those where it was less than 50% expression was considered as negative for statistical analysis in this study.

3.2.5 Cell Cycle analysis (Ploidy and S phase fraction estimation) protocol:

5-10 mg of fresh/frozen tissue was taken in a Petri dish submerged with hypotonic Propidium iodide (PI) solution (Sigma Aldrich, USA) (50 µg/ml). It was minced firmly with a scissor to prepare single cell suspension solution and then collected in a polypropylene tube for centrifugation at 2000 rpm for 5 minutes. It was washed with phosphate buffered saline a single time, filtered with 39 micron nylon filter, centrifuged at 2000 rpm for 5 minutes and supernatant was discarded. Fresh 500 µl of PI was added and incubated in the dark for 20 minutes.

The sample was acquired on flow cytometer (Beckton Dickinson, USA) with 488 nm laser and with the help of Cell Quest software. The data was analyzed with Mod Fit software, USA. A schematic diagram of cell cycle experiment listed in figure 11.

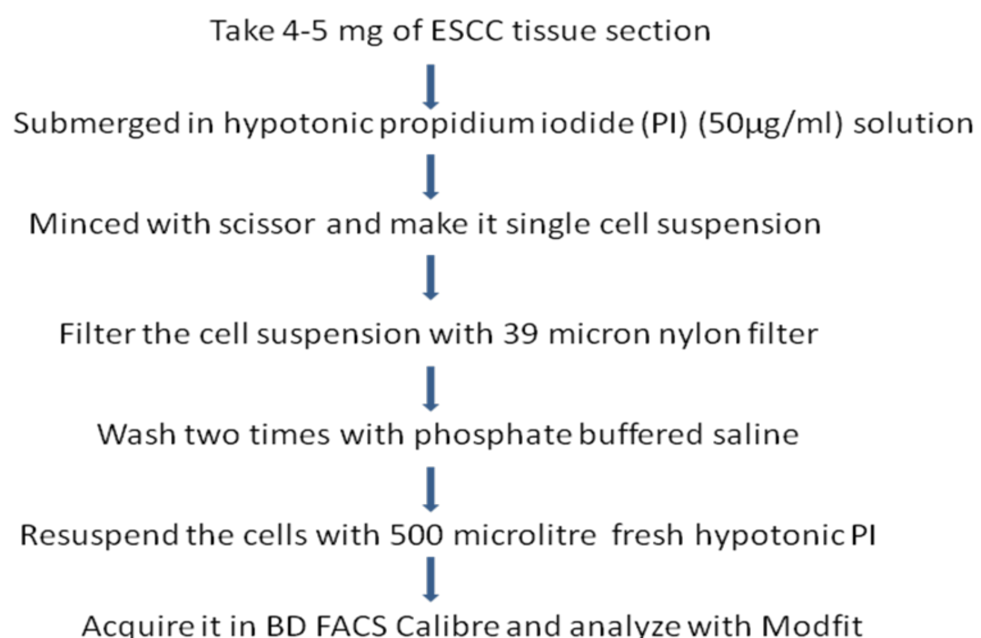

Figure 11. A schematic diagram of cell cycle experiment.

3.2.6 Statistical analysis:

$\chi 2$ with likelihood ratio (LR) or Fisher's exact test was used for correlation analysis. Software SPSS version 10 was utilized for statistical analysis. In all tests, 2 tail *P* value was used and test was considered statistically significant when $P \leq 0.05$. For the analysis of overall survival, Kaplan-Meier probability distribution test was used.