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ALTERATION IN PROTEIN EXPRESSION OF AURORA A, FHIT AND IGF2 IN ESOPHAGEAL CANCER

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ABSTRACT

Evaluating protein levels, especially of those related to cell cycle and mitotic machinery, can be effective in cancer detection and therapeutics. The protein expression of Aurora A, FHIT, and IGF2 were evaluated in esophageal cancer. 51 subjects were included: squamous cell carcinoma (n=40), adenocarcinoma (n=8) and normals (n=3). Immunohistochemistry was performed on tissue sections obtained from archival esophageal tissue blocks to evaluate protein expression. Immunohistochemistry with active Aurora A (phosphoT288) showed moderate-high cytoplasmic expression in 100% normal cases [mean:2.16(±0.28)], whereas only 37.5% of cancers showed mild-moderate Aurora A expression [mean:0.51(±0.70)]. Aurora A expression showed four-fold decrease in cancer when

compared to normal esophageal tissue. IGF2 (cytoplasmic) expression was observed to be mild-moderate in all normal samples. Of the cancers, 81.2% showed IGF2 expression where mean expression of IGF2 in normal tissue was 0.57(±0.28), while in cancer, it was 1.29(±0.77). IGF2 expression showed a two-fold increase in cancers when compared to normal tissue. This could be attributed to loss of imprinting, which was however not investigated. FHIT expression showed mild (0.4-fold) decrease in cancer tissue when compared to normal; however this did not seem significant. Our results show that reduced active form of Aurora A expression was significantly associated with esophageal cancer. It maybe a good marker to predict cellular progression to cancer in the esophagus. Active form of Aurora A expression should be taken into account before the use of kinase inhibitors in esophageal cancer therapy. Loss of FHIT expression and increased IGF2 may also contribute to cancer; FHIT expression did not seem significant.

Keywords: Esophageal Cancer, Aurora A, Fragile Histidine Traid, insulin like growth factor 2.

1. INTRODUCTION

We ask that authors follow some simple guidelines. In essence, we ask you to make your paper look exactly like this document. The easiest way to do this is simply to download the template, and replace the content with your own material. The events underlying the transformation of normal esophageal epithelia to malignant tumour cells are poorly understood even though genetic abnormalities in several oncogenes and tumour suppressor genes are known to occur frequently in esophageal cancer (1-3). Approaches to targeted cancer therapy have to be designed and can effectively be put to use, after evaluating the expression status of several of these molecules.

Aurora A, also known as STK15, BTAK, or AURKA located on chromosome 20q13.2, a member of the serine/threonine kinase family, is a centrosome-associated protein implicated in regulating centrosome function, spindle assembly, maintenance, chromosome segregation and cytokinesis. Accurate segregation during mitosis is important for normal cell division. Check-point controls and other mitotic events are tightly regulated by phosphorylation and dephosphorylation of different proteins. Suppression of Aurora A results in defects in the mitotic process, incomplete cytokinesis and genomic instability; its dysregulation has been associated with cancers in different tissues like breast, lung, head and neck, glioma and colon (4, 5).

The fragile histidine triad (FHIT) gene (3p14.2) is composed of 10 exons that encompass a 1.8 Mb genome region, a tumour suppressor that is frequently inactivated by alterations of the FHIT transcripts in a variety of human cancers, including lung, colon, esophageal, breast, head and neck, stomach, cervical and pancreatic carcinomas (6-11). Specific chromosomal translocations disrupt a fragile site, FRA3B. The highest frequency of allelic deletions were observed in lung cancer tumors linked to carcinogenic exposure, inducing susceptibility to breakage at 3p site. FHIT is reportedly inactivated in epithelial tumors, particularly in tumors resulting from exposure to environmental carcinogens like smoking and exposure to asbestos (12). Urashima *et al.*, (2013) investigated the effects of alcohol consumption on somatic copy-number alterations (SCNAs) across the whole genome which included FHIT as a finding (13). The present study evaluated altered expression of FHIT in esophageal cancer from our population.

The gene encoding IGF2 (11p15) is an imprinted gene where an epigenetic modification leads to differential expression of the two parental alleles of the gene in somatic cells of the offspring (14). Increased cellular proliferation and an increased sensitivity of the IGF2 signaling pathway, leading to an increased risk for gastrointestinal neoplasia and premalignancy, has been associated with loss of imprinting (LOI) (15). Aging is one of the processes where the dysregulation of the differentially methylated regions on the maternal chromosome causes LOI and overexpression (16). The MAPK pathway appears to be the main pathway whereby IGF2 and other ligands of IGF1R activate genes concerned with cell proliferation causing mitogenesis. PI3-K/Akt pathway is also activated, leading to reduced apoptosis and increased cell survival (17). Li *et al*., (2014) provided evidence to show that Id1 overexpression could promote IGF2 over-secretion, which activates AKT in an autocrine manner to push cell proliferation and invasion. In the present study, IGF2 expression was evaluated in esophageal cancer (18).

2. MATERIALS AND METHODS

2.1 Sampling

A total of 51 subjects were included in the present study having esophageal squamous cell carcinoma (n=40), adenocarcinoma (n=8) and normal tissue (n=3); archival esophageal tissue blocks were selected, tissue sections were obtained from Department of pathology, Kamineni Hospitals. Ethics clearance was obtained from Vasavi Institutional Ethics committee, VMRC, Hyderabad.

2.2 Immunohistochemistry

Protein expression was evaluated by immunohistochemistry method manually for Aurora-A, FHIT and IGF2 genes by the method published earlier from our group (19). Protocol in brief, 4µm thick tissue sections were taken on to the gelatin chromalum coated slides and tissue fixation was performed. Deparafinization was done at 70^{o}c for 60 min in hot-air oven followed by 3 xylene washes each 5min. Tissue slides were further processed for gradient alcohols 5min each and subsequently in running tap water for rehydrating the tissue. Antigen retrieval was performed by trisodium citrate buffer (pH-6) in microwave oven (700watts) for three cycles and left out at room temperature for cooling for 30min. Two PBS (Phosphate buffer saline, pH-7.5) washes given to the tissue slides (5 min each) and endogenous peroxidase blocking was done by applying peroxidase block solution for 10min (Provided in Expose Rabbit specific HRP/DAB detection IHC Kit (ab80437; Abcam, Cambridge Science Park, UK), rinsed with two PBS washes and protein block applied to the section incubated for 10min. Primary Abs (Rabbit polyclonal Aurora-A-ab58494, 1/50; FHIT-ab15287, 1/50 and IGF2-ab9574, 1/100 dilution; Abcam, Cambridge Science Park, UK) applied so that entire tissue section gets covered and incubated in moisture chamber for 2hrs. After incubation PBS washes given to remove unbound primary Ab. HRP conjugated secondary Ab applied for 15 min followed by two PBS washes and DAB chromogen (10 min) applied to see the colour product. Slides were rinsed with deionized water and counter stain (Hematoxylene) applied for 2min and then slides were transferred to running tap water for 5 min. Slides cleaned and dried mounting was performed with DPX mount. One negative control without primary Ab in each experiment was kept. Screening was performed as described earlier from our group (20). Data was categorized based on the histology/pathological type and statistical analysis performed.

Semi quantitative scoring was performed at a magnification of ×400. From each section five regions were scored based on the intensity and percentage of stained cells; expression score was recorded as follows; 0.5 negative, 1 mild, 2 moderate, 3 high and 4 intense expression. Two independent scorers, blinded to the type of histological specimen, performed the analysis; any discrepancies were resolved by discussing with the pathologist.

3. RESULTS

A total of 51 subjects were included in the present study; 31 males and 20 females with mean age of the cases 59.3yrs ± 15.1. Based on histopathology, cases were categorized as normal, adenocarcinoma (ADC) and squamous cell carcinoma (SCC).

3.1 Aurora A expression

IHC showed endogenous levels of Aurora A (phosphoT288, cytoplasmic) active form in the different esophageal tissue (Figures. 2, 3 & 4). Moderate-high expression of active form of the Aurora A was observed in 3/3 (100%) normal samples.

Expression of Aurora A was seen in 18/48 (37.5%) cancer samples; of these, 9/18 (50%) with mild expression, 5/18 (27.8%) with moderate expression, 2/18 (11.1%) with high expression and 2/18 (11.1%) showed intense expression of Aurora A. Rest of the 62.5% tumor tissues showed very poor Aurora A staining and were scored as negative. Difference of expression in ADC and SCC tissue is given in Table 1. Mean expression of Aurora A in normal tissue was 2.16 (±0.28), in cancer tissue, it was 0.51 (± 0.70). Aurora A expression was four-fold reduced in cancers when compared to normal esophageal tissue [Figure.1 (1A &1B)].

Table 1. Aurora A protein expression in esophageal cancer and normal tissue

SNO	Pathology (n)	Positive Expression +ve /n (%)	Mild Expression	Moderate Expression	High to Intense Expression	Mean Expression (SD) Aurora-A
1	**Normal (3)**	3/3 (100)	-	1/3 (33.4)	2/3 (66.4)	2.16 (± 0.28)
	Cancer (48)	18/48 (37.5)	9/18 (50)	5/18 (27.8)	4/18 (22.2)	0.51 (± 0.70)
2	**Adenocarcinoma (8)**	4/8 (50%)	2/4 (50)	1/4 (25)	1/4 (25)	0.65 (± 0.73)
3	**Squamous cell carcinoma (40)**	14/40 (35)	7/14 (50)	4/14 (28.6)	3/14 (21.4)	0.49 (± 0.70)

i	**WDSCC (14)**	6/14 (42.8)	4/6 (66.7)	2/6 (33.3)	-	0.36 (± 0.33)
ii	**MDSCC (12)**	3/12 (25)	2/3 (66.7)	1/3 (33.3)	-	0.33 (± 0.32)
iii	**PDSCC (14)**	5/14 (35.7)	1/5 (20)	1/5 (20)	3/5 (60)	0.75 (± 1.0)

3.2 IGF2

IGF2 (cytoplasmic) was expressed mildly in 2/3 (66.6%), and moderately in 1/3 (33.3%) in normal esophageal tissue.

Of the cancer samples, 39/48 (81.2%) showed IGF2 expression where 6/39 (15.4%) had mild expression, 14/39 (35.9%) with moderate expression, 18/39 (46.1%) with high expression and 1/39 (2.6%) showed intense expression of IGF2. Table 2 shows the expression in ADC and SCC (Figs. 2, 3 & 4). Mean expression of IGF2 in normal tissue was 0.57 (±0.28), while in cancer tissue, it was 1.29 (±0.77). IGF2 expression showed a two-fold increase in esophageal cancers when compared to normal esophagus [Figure.1 (1C & 1D)].

Table 2. IGF2 protein expression in esophageal cancer and normal tissue

SNO	Pathology (n)	Positive Expression +ve/n (%)	Mild Expression	Moderate Expression	High to Intense Expression	Mean Expression (SD)IGF2
1	**Normal (3)**	3/3 (100)	2/3(66.7)	1/3(33.3)		0.57 (0.28)
	Cancer (48)	39/48 (81.2)	6/39 (15.4)	14/39 (35.9)	19/39 (48.7)	1.29 (0.77)
2	**Adenocarcinoma (8)**	8/8 (100)	1/8 (12.5)	3/8 (37.5)	4/8 (50)	1.53 (0.66)
3	**Squamous cell carcinoma (40)**	31/40 (77.5)	5/31 (16.1)	11/31 (35.5)	15/31 (48.4)	1.24 (0.79)
i	**WDSCC (14)**	10/14 (71.4)	1/10 (10)	4/10 (40)	5/10 (50)	1.21 (0.76)
ii	**MDSCC (12)**	10/12 (83.3)	3/10 (30)	4/10 (40)	3/10 (30)	1.15 (0.76)
iii	**PDSCC (14)**	11/14 (78.5)	1/11(9)	3/11 (27.3)	7/11 (63.7)	1.36 (0.89)

3.3 FHIT

FHIT was highly expressed in 3/3 (100%) normal esophageal tissue samples. In esophageal cancer tissue, 41/48 (85.4%) showed FHIT expression, of which 7/41 (17%) had mild expression, 18/41 (44%) showed moderate expression, 13/41 (31.7%) showed high expression and 3/41 (7.3%) had intense expression of FHIT (Figures. 2, 3 & 4). Expression differences between ADC and SCC cases is given in table 3 & fig. 1 (1E & 1F). Mean expression of FHIT in normal tissue was 1.85 (±0.32), in cancers, it was 1.30 (±0.77). FHIT expression showed a 0.4-fold decrease in cancers when compared to normals which was not seen as significant. However, when we compared the high expression in all the normal esophageal tissue samples to 84.5% of the cancers that scored positive for FHIT expression, it was observed that 61% cancers showed mild expression as against 39% cancers which showed high expression, indicating a reduction in the expression of this protein in cancer tissue (Figures. 3 & 4).

Table 3. FHIT protein expression in esophageal cancer and normal tissue

SNO	Pathology (n)	Positive Expression +ve/n (%)	Mild Expression	Moderate Expression	High to Intense Expression	Mean Expression (SD) FHIT
1	**Normal (3)**	3/3 (100)	-	-	3/3 (100)	1.85 (0.32)
	Cancer (48)	41/48 (85.4)	7/41(17)	18/41(44)	16/41(39)	1.30 (0.77)
2	**Adenocarcinoma (8)**	8/8 (100)	-	3/8 (37.5)	5/8 (62.5)	2.02 (0.66)

3	**Squamous cell carcinoma (40)**	33/40 (82.5)	7/33 (21.2)	15/33 (45.5)	11/33 (33.3)	1.15 (0.71)
i	**WDSCC (14)**	13/14 (92.8)	3/13 (23.1)	7/13 (53.8)	3/13 (23.1)	1.14 (0.52)
ii	**MDSCC (12)**	8/12 (66.7)	1/8 (12.5)	5/8 (62.5)	2/8 (25)	0.92 (0.72)
iii	**PDSCC (14)**	12/14 (85.7)	3/12 (25)	3/12 (25)	6/12 (50)	1.36 (0.85)

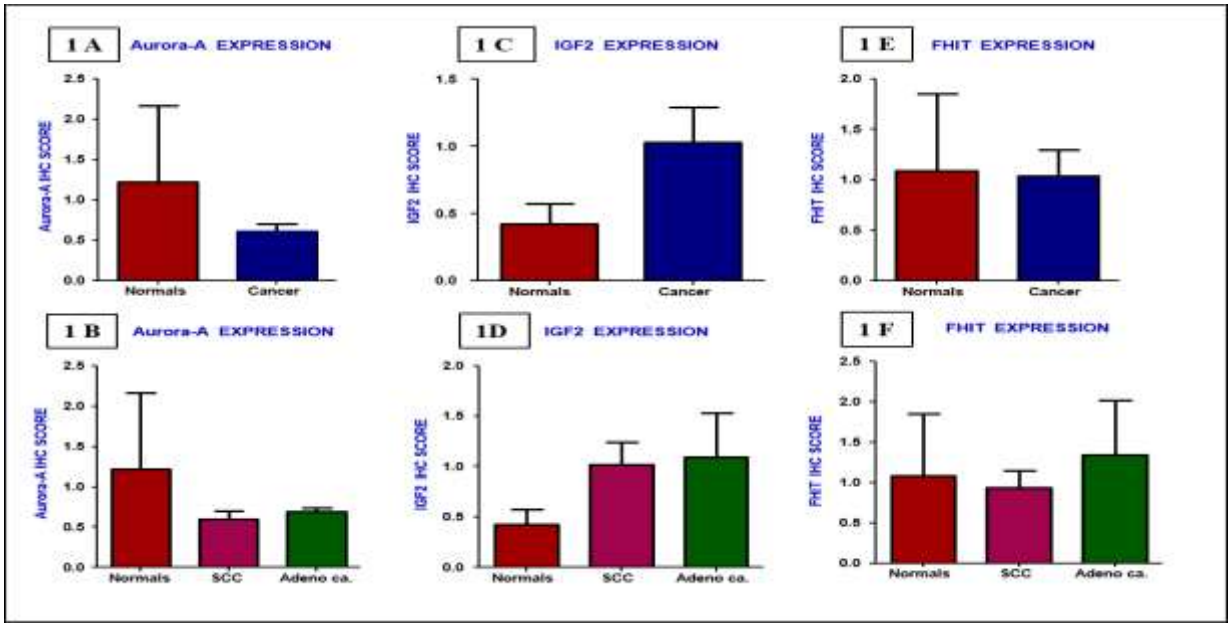

Fig 1.: Mean expression of phosphorylated Aurora A (1A, 1B), Mean expression of IGF2 (1C,1D) and FHIT (1E, 1F) in esophageal tissue.

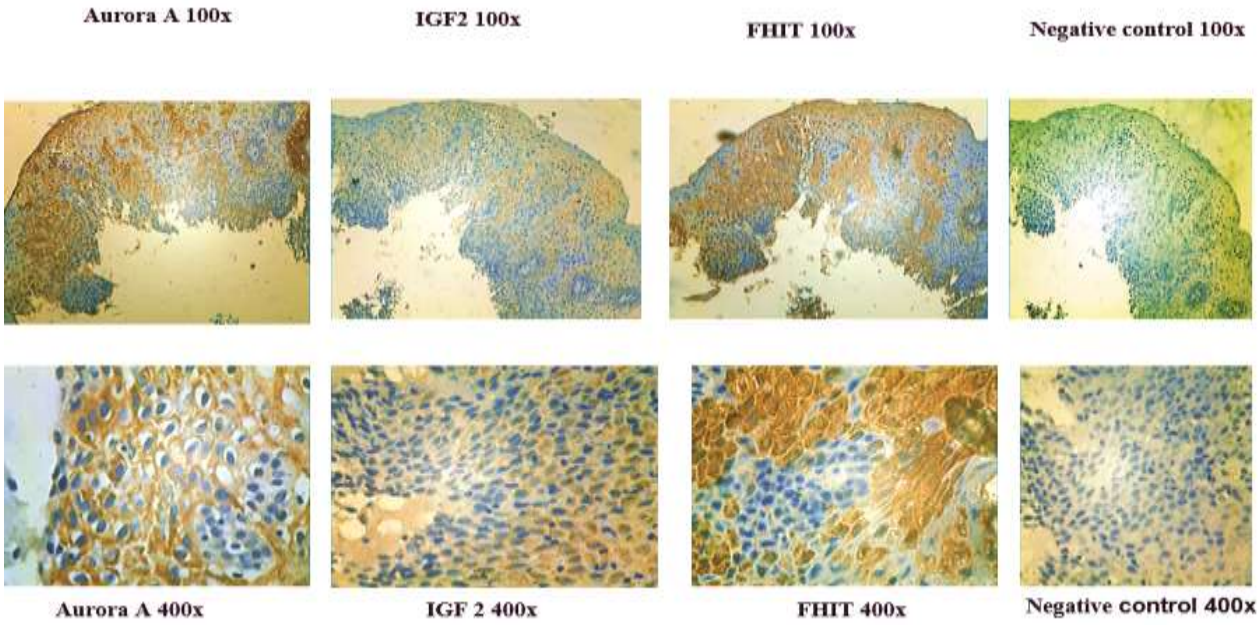

Fig 2. Aurora A, IGF2 and FHIT expression in Normal Esophageal Tissue

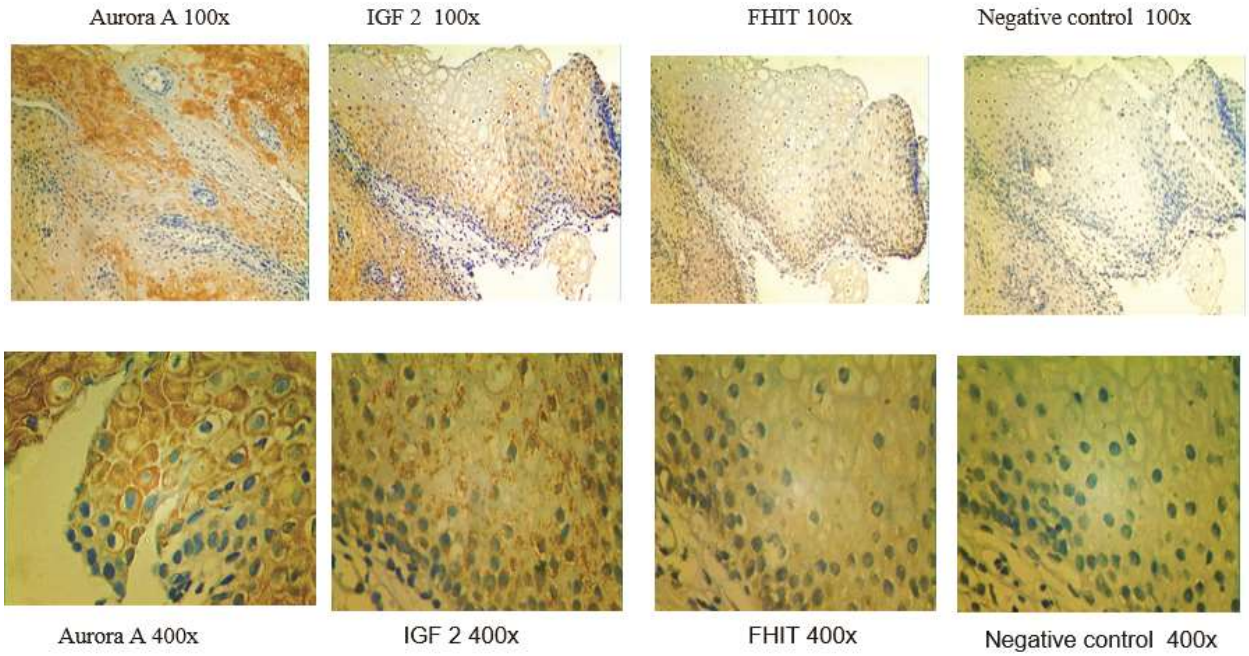

Fig 3. Aurora A, IGF2 and FHIT expression in Esophageal Squamous cell carcinoma

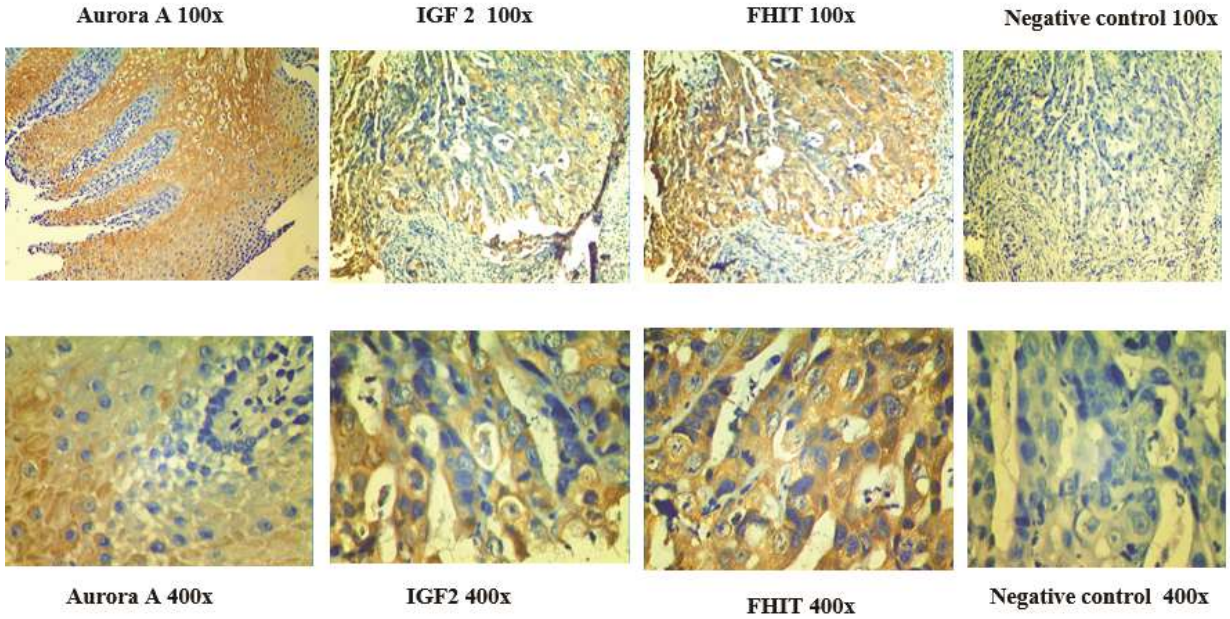

Fig 4. Aurora A, IGF2 and FHIT expression in Esophageal Adenocarcinoma

4. DISCUSSION

Esophageal cancer ranks among the ten most frequent cancers in the world, with a five-year survival rate of less than 10% (21). It is very important to identify molecular changes that can be targeted for therapy to improve treatment outcome. The actual development of the disease phenotype has been associated in large part, with the individual's environment and lifestyle. Epidemiological studies have revealed that the incidence of esophageal SCC is associated with several environmental risk factors, such as tobacco smoking, heavy alcohol drinking, micronutrient deficiency and dietary carcinogen exposure (22-24). Genetic abnormalities in ESCC and esophageal cancer cell lines have been reported by many workers (1-3). In the present study, we aimed to investigate the expression of Aurora A, IGF2 and FHIT to identify if they could be potential targets for therapy.

Aurora A kinase regulates the functions of centrosomes, spindles and kinetochores and is necessary for proper separation of the centrosomes after the mitotic spindle has been formed. It helps orchestrate an exit from mitosis by contributing to the completion of cytokinesis (25, 26). Aurora A is transcriptionally upregulated during mitosis and degraded after metaphase by the ubiquitin-mediated proteasome, which is promoted by the hCdh1-activated anaphase promoting complex/cyclosome (27). Its activity is regulated by phosphorylation (autophosphorylation or by PKA) and dephosphorylation (28, 29). Thr288 phosphorylation is important in regulating the level of Aurora A kinase activity. Aurora A complexes with PP1c (Protein phosphatase 1c). Indirect pathways such as the inhibition of PP1 by Cdc2-cyclin B may act for the activation of Aurora A kinase (30). Aurora A inactivated by DNA damage (at the end of G2) or insufficiently activated (Thr288phos) Aurora A, may resulted in defective bipolar spindle formation and chromosomal segregation, thereby leading to aneuploidy (31).

Some studies indicate that overexpression of Aurora may be due to the amplification at 20q region or upstream regulatory proteins (32, 33). It was reported to be overexpressed in various cancer cells, including esophageal cancer (25, 34-37). Overexpression of Aurora A abrogates G2 checkpoint in Rat1 cells. Aurora A phosphorylation of S315 on p53 increases MDM2-dependent degradation of p53. Normally, p53 interacts with the Aurora A A-box (mutation at Serine 53 residue leads to accumulation) and inhibits its kinase activity; that is, p53 guards against the potential for transformation when Aurora A is overexpressed. This homeostatic relationship is deregulated in cancer (38, 39). In our cases, since Aurora A showed reduced expression, it is assumed that this activity of p53 is unaffected.

In the present study, the mean expression of Aurora A in esophageal cancer tissue (0.51) was significantly decreased when compared to expression in normal tissue (2.16). While all the normal cases showed significant expression of the active form, only 50% ADC and 35% SCC expressed the Thr288phos Aurora A protein. (It was observed that in poorly differentiated squamous tissue and in ADC, expression was a bit higher than in the other cancers, though reduced compared to normal esophageal tissue). The four-fold decrease in phosphorylated Aurora A may be affecting the spindle apparatus during mitosis and promoting chromosomal anomalies in the tumor tissue. According to earlier work from our lab, 82% of cancer tissues were found to be methylated at Aurora A promoter, compared to 31% normal esophageal tissue. The decreased expression observed by us can be attributed to methylated gene promoter, contributing to aneuploidy and malignancy in the esophagus (40, 41).

Aurora A alone is not a potent inducer of malignant transformation. Furukawa *et al*., (2006) reported downregulation of the MAPK pathway affected the expression of Aurora A protein. Profiling of gene expressions associated with downregulation of MAPK1 induced by exogenous overexpression of DUSP6 gene in pancreatic cancer cells, showed that AURKA gene was among the downregulated genes. Inhibitors of kinase activity are often thought of, as strategy for treating esophageal tumors. Expression of Aurora A should be evaluated before considering such therapeutic intervention (33).

Homozygous deletions in FHIT have been observed in a variety of human tumors (42); loss of heterozygosity or hypermethylation of the CpG island may affect the expression of the protein (43). Ohta *et al.*, (1996) reported that FHIT gene abnormalities often occur in primary digestive tract cancers (7).

High FHIT expression was observed in normal samples whereas 85.4% cancers [1.30 (±0.77)] showed lesser FHIT expression than normal esophageal tissue [1.85 (±0.32)]. The intensity of expression was observed to be high in the normal tissue, whereas in cancer samples, 61% showed mild to moderate, and a lesser (39%) number of samples showed high to intense expression. Though there was 0.4-fold decrease in cancers, the mean expression did not showed considerable difference. Expression differences between ADC and SCC seem to be more random. From this study, FHIT expression may not be significantly associated with disease, though there is some reduction in expression from normal esophageal tissue to cancer tissue.

FHIT is susceptible to environmental changes. Exposure to benzo[α]pyrene diol epoxide (BPDE), a carcinogen present in tobacco smoke and environmental pollution in general, has been shown to be associated with promoter methylation of the FHIT gene in esophageal SCC (44). Carcinogens cause damage at FRA3B, leading to loss of exons and a loss of the second FHIT allele leads to total loss of FHIT protein expression (45). A study to look at environmental, genetic and epigenetic alterations and correlate with its expression in esophageal cancer will give a clearer view with respect to its role.

Genomic imprinting is a non-Mendelian inherited epigenetic form of gene regulation (17). LOI of IGF2 results in increased transcription of this growth promoter (46-48). LOI was reported in multiple cancers including esophageal cancer (49). In the present study, IGF2 expression was found to be mild to moderate in normal samples. 81.2% cancer cases stained positive for IGF2 expression; ~50% of these showed high IGF2 expression. Mean expression of IGF2 in normal esophageal tissues was 0.57 (±0.28), while in cancers, it was 1.29

(±0.77); this is in accordance with others' reports that IGF2 showed two-fold increased expression in cancers when compared to normal tissue (47, 48). Here, IGF2 was elevated in a greater % of cases with ADC and poorly differentiated esophageal cancer indicating it to be a marker for poor prognosis; targeted therapy may greatly benefit this group of patients.

Elevated expression of growth factors will stimulate the cancer cells to become more aggressive. Targeting this elevated expression of IGF2 using neutralizing antibodies, and its receptor IGF1R, may be an effective therapeutic strategy. IGF1R blockade has been known to elevate the chemosensitivity of cancer cells in animal models. A number of IGF1R inhibitors (cixutumumab helps cancer cells overcome 5-FU resistance) are currently in clinical trials for the treatment of solid tumors (18, 50).

In conclusion, our results showed that reduced phosphorylated Aurora A expression and increased IGF2 may play a role in esophageal carcinogenesis. Reduced phosphorylated Aurora A expression may be a good marker to predict cellular progression to cancer. IGF2 protein expression may be playing a role as a mitogen promoting proliferative changes in esophageal tissue and driving carcinogenesis. Loss of FHIT expression may be important when considering exposure to carcinogens (environmental/dietary) in the esophageal tissue.

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