Original Article

Mutations in Epidermal Growth Factor Receptor Gene in Esophageal Squamous Cell Carcinoma Patients in Kashmir- a High Incidence Area of India

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Abstract

Activating mutations in Epidermal Growth Factor Receptor (EGFR) are common in lung adenocarcinoma of never smokers but are rare in other types of cancer. Here we have analysed mutations in exons 19 to 21 of EGFR and in exons 19 and 20 of the EGFR homolog HER2 in 54 cases of Esophageal Squamous Cell Carcinomas (ESCC) from patients recruited in Kashmir, India, a region of high incidence for this cancer. We report the detection of 3 mutations (6%) in the ATP-binding regulatory loops of the tyrosine kinase domain of EGFR (deletion 746-750, P753L, G719D). No mutation was found in HER2. This is the first report of activating EGFR mutations in ESCC, of the same type as those detected in lung adenocarcinoma of never-smokers. This suggests that a small proportion of ESCC patients in this high incidence area may benefit from treatment with EGFR tyrosine kinase inhibitors.

Key words : EGFR, Mutations, Esophageal Cancer, Kashmir

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Introduction

Esophageal cancer is the eight most common cancer and sixth cause of cancer deaths worldwide, with the majority of cases occurring in developing countries [1]. There are striking geographic variations in incidence. Very high incidence rates (over 40-50/100,000/year in males and in females) have been consistently reported in a region that extents from the Caspian Sea to Central Asia, defining the so called "*Esophageal Cancer Belt*" [2]. Kashmir Valley, in Northern India, lies at the south border of this high incidence region. Although there is no continuous cancer registration in Kashmir, current observational studies indicate that Kashmir Valley is a region of high risk of esophageal

cancer [3;4], with incidence of 42 and 27/100.000/year for men and women, respectively [5]. Independently of geographic origin, the development of ESCC is accompanied by genetic alterations that include frequent loss of alleles at chromosomes 3p, 5q, 9p and q, 13q, 17p, 17q or 18q [6], mutations in tumor suppressor genes such as *TP53,* [7], and diverse genetic and epigenetic alterations genes such as *CDKN2a, CCDN1, MYC1 FHIT, FEZ1, DLC1, Annexin-1, CCNB1, TP63, TP73* or *DCC [8-13]*.

So far, there is only very limited information on the status of EGFR and HER2 in ESCC. Both genes belong to the ERBB family of transmembrane receptors. There is evidence that EGFR1 is often over expressed and some times amplified in ESCC but no systematic study has addressed whether EGFR may carry activating mutations. Such mutations are common in lung carcinomas of never-smokers, which are predominantly adenocarcinomas (ADC). They have attracted considerable clinical interest due to their association with tumour sensitivity to the antiproliferative effects of tyrosine kinase inhibitors, erlotinib and gefintinib [14;15]. Most of described *EGFR* mutations (85%) fall into two categories: point mutations in exon 21 (L858R; 40%), and in-frame deletions of 2 to 9 residues in exon 19, encompassing residues of a conserved LREA motif (residues 748-751) (45%) [16]. Other mutations include rare insertions in exon 20, a minor hot spot at exon 18 (5%) and scattered missense mutations in exons 18 to 21. There is structural and biochemical evidence that the L858R mutation and the short deletions in exon 19 modify the geometry of the ATP binding cleft in the tyrosine kinase (TK) of *EGFR*, resulting in a hyperactive form of the receptor [16]. *EGFR* mutations are inversely correlated with tobacco consumption and are reported to be more frequent in ADC of women of Asian descent than in Caucasian [17;18]. *HER2* is a member of the *EGFR* family that is dysregulated in many cancers. The most common genetic alteration of *HER2* is amplification leading to over expression, which is frequent in breast and ovarian cancers and is associated with poor prognosis [19;20]. *HER2* can dimerise with other members of the ERBB family and current evidence suggests that HER2 mediates most of these effects by forming a heterodimer, *EGFR/HER2*. Recent studies have reported that mutations in the TK domain of *HER2* in a small subset of lung cancers [18]. In the present study, we have analyzed *EGFR* (exon 18 to 21) and *HER2* (exons 19 and 20) mutations in 54 ESCC from Kashmir Valley.

Materials and Methods

Study subjects and tumors

Patients presenting for treatment of ESCC for the first time (n=55) at the Departments of Cardiovascular and Thoracic Surgery and Gastroenterology of the Sher-i-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir, between July 2002 and July 2003 were recruited for the study, with prior informed consent. Surgically resected samples from 17 patients diagnosed with ESCC and 38 biopsy specimens obtained at endoscopy from patients complaining of dysphagia and histopathologically confirmed as ESCC were collected

Information on suspected risk factors such as salt tea intake and information regarding tobacco use (water pipe smoking and snuff) were obtained using *adhoc* questionnaires. These data are summarized in Table 1.

Mutation Analysis

DNA was extracted from all but one resected tissue or biopsy (54 patients). *EGFR* mutations were detected using PCR-based direct sequencing of the four exons of the TK domain (exons 18-21) using primers and annealing conditions as described by Pao *et al* [15]. *HER2* was amplified using the following sense and antisense primers for exon 19 and 20: 19F (5'GGATCCAGCCCACGCTCTT3') and 19R (5'CTGCAGCCATGGGGTCCTT3'); 20F (5'CCATACCCTCTCAGCGTA3') and 20R (5'GCTCCGGAGAGACCTGCAA3') [18]. Amplifications were performed by using a touch-down protocol from 65°C to 62°C for exon 19 and from 61°C to 58°C for exon 20. Aliquots of PCR products were examined by electrophoresis on 2% agarose gel containing ethidium bromide. PCR products were treated with 2µl of ExoSAP-IT (Amersham Biosciences, Piscataway, NJ) at 37°C for 15 minutes followed by inactivation at 80°C for 15 min and direct sequencing using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer, Foster City, CA) on ABI PRISM 3100 Genetic Analyser (Applied Biosystem, Foster City, CA).

Results

In this study we have analysed EGFR mutations in exons 18-21 by direct sequencing in 55 archival cases of ESCC from patients recruited at the Departments of Cardiovascular and Thoracic Surgery and Gastroenterology of the Sher-i-Kashmir Institute of Medical Sciences, Soura, Srinagar, Jammu and Kashmir, between July 2002 and July 2003 (Table 1). The mutation status of TP53 in these tumours has been reported previously [21]. All specimens but one provided sufficient good quality DNA for PCR and sequencing. A total of 3 mutations in *EGFR* (exons 18 to 21) were found in 54 analysable ESCC. These mutations were one 15 bp deletion in exon 19 (codons 746-750 ; case T35) and two missense mutations, at codon 753 (exon 19 ; CCG to CTG, P to L; case T3) and 719 (exon 18, GGC to GAC, G to D, case T53) . Two synonymous polymorphisms were detected, one at codon 836 (exon 21, CGC to CGT, R to R ; case T 47), and the other at codon 787 (CAG to CAA, in 40 of 54 evaluated cases). Presence of an EGFR mutation was independent of TP53 mutation status. No mutations were found in exons 19 and 20 of HER2, encoding the tyrosine kinase domain of receptor closely related to EGFR. Of the patients analysed here, 20 were previously reported to carry a TP53 mutation. Only one patient (T53) was found to have both *EGFR* and TP53 mutation.

Discussion

Mutations in the Epidermal Growth Factor Receptor (EGFR) have attracted attention because of their common occurrence in lung cancers of never smokers, in particular in adenocarcinoma and in women (20 to 40%). There is now good evidence that the most frequent of these mutations are activating the receptor's Tyrosine Kinase activity. These mutations are often associated with therapeutic response to drugs inhibiting the receptor tyrosine kinase (TKI) such as gefitinib or erlotinib [14;15]. A total of 2791 mutations are currently compiled in the COSMIC mutation database, 2696 of them detected in lung cancers. Mutations are exceedingly infrequent in other cancer pathologies analyzed to date [22]. EGFR has a critical role in the morphogenesis, proliferation, differentiation and

renewal of many epithelial tissues including the esophageal mucosa. In ESCC, EGFR1 amplifications and over expression have been reported in advanced cancers [23;24]. However, so far there is no report on EGFR mutations in primary ESCC.

Here we report the presence of mutations in the TK domain of EGFR in a total of 3 ESCC patients. Two of the three mutations were found in exon 19: one small deletions of 15 base pairs (746-750) and one point mutation, P753L. The third one, G719D, was detected in exon 18. None of the patients presented a mutation of HER2.

In frame deletions encompassing codons 746-750 and missense mutations at codon 719 are common types of EGFR mutation in lung cancers [18]. They alter domains of the kinase known as the A loop (activation, a highly conserved region encompassing a LREA motif) and the P loop (phosphate, a glycine-rich sequence located in the ATP-binding cleft). Both domains play crucial roles in ATP binding and kinase activation, and both mutant proteins have been experimentally shown to be sensitive to inhibition by TKI. Mutations at codon 753 have also been reported in lung cancer. This residue is directly flanking the conserved motif in the A-loop [16]. However, its functional impact on kinase activity has not been assessed experimentally.

In lung cancers, EGFR mutations are preferentially, if not exclusively, found in tumors of never-smokers. These tumors are mainly adenocarcinomas, although EGFR mutations have occasionally been reported in lung squamous cell carcinoma. Furthermore, most of these mutations are found in women. In the present study, the patients are not cigarette smokers but are users of tobacco in other forms, mostly “hukka” (smoking water pipe) and/or snuff (Table 1). Of the three patients with EGFR mutations, one is a female non-smoker and two are male with reported high daily hukka use. There was no obvious association with any exposure or clinical parameter. Thus, although limited, our results do not suggest that EGFR mutations in ESCC are distributed with the same exposure and gender biases as in lung cancers.

This is the first report that mutations in EGFR similar to those detected in lung cancers of never-smokers may also occur in primary ESCC. So far, only 7 EGFR mutations have been reported in esophageal cancer, all in adenocarcinomas. In ESCC, Hanawa et al. [23] did not detect any mutation in 40 cases, including 15 cases with EGFR amplifications. Guo et al. [25] described a gefinitib-sensitizing mutation in the ESCC-derived KYSE450 cell lines, and found 3 mutations in 57 primary ESCC. These mutations included two silent substitutions and a truncation at codon 872. Whether the latter induces constitutive activation of the kinase is not known, but this is unlikely given the fact that regions downstream of this residue are important for EGFR kinase activity and signal transduction.

Kashmir belongs to the high ESCC incidence area of central Asia. The incidence of ESCC in this area is 10 to 100 higher than in many other regions of the world. The occurrence of EGFR mutations in a small subset of patients from a high incidence suggests that the use of TKI inhibitors may represent a potential therapeutic intervention in ESCC. Since treatment options are very limited in high-incidence, low to middle resource countries, such an intervention may have a significant clinical impact.

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Table 1: Characteristics of patients, tumours and EGFR mutations

Patient Number	Age[1]	Sex[2]	Tobacco use[3]	Snuff use[4]	Salt tea[5]	Tumor Site[6]	Grade[7]	EGFR Mutation
T1	50	M	+	+	6	M	M	-
T2	52	M	++	-	6	M		-
T3	35	F	-	-	6	M	M	Exon 19, CGC to CTG P753L
T4	56	M	+++	-	6	L	W	--
T5	50	F	+++	-	12	M	P	-
T6	50	M	+++	-	9	M	P	
T7	45	M	+++	-	20	L	M	-
T8	60	M	+++	-	20	L	M	-
T9	65	M	+++	-	18	M	PD	-
T10	53	M	2++	-	NA	M	M	-
T11	55	M	+++	-	3	L	P	-
T12	50	F	+	-	12	M	P	-
T13	40	F	-	-	12	L	W	-
T14	62	M	+++	-	12	M	P	
T15	60	F	+++	-	NA	L	M	-
T16	75	M	+++	+	9	L	P	-
T17	50	M	++	-	12	L	W	-
T18	55	M	++	-	9	L	P	-
T19	45	F	-	-	NA	M	M	-
T20	70	M	+++	+	9	M	M	Not Done
T21	55	M	+++	-	6	L	M	-
T22	65		+++	-	10	M	M	-

T23	60	F	++	-	12	M	M	-
T24	65	F	-	-	8	M	M	-
T25	60	F	-	+	12	M	W	-
T26	65	M	+	+	15	M	W	-
T27	65	F	-	+	9	M	M	-
T28	55	M	++	-	9	M	M	-
T29	70	M	+++	-	12	L	M	-
T30	60	M	+++	+	12	L	P	-
T31	60	M	+++	-	9	L	M	-
T32	65	F	-	+	9	M	M	-
T33	70	M	++	-	9	U	M	-
T34	47	M	++	+	6	L	M	-
T35	70	M	+++	-	NA	L	M	Exon 19;15BP,Deletion Codon 746 – 750;
T36	50	M	++	-	6	M	P	-
T37	60	F	-	-	15	M	M	-
T38	60	F	++	+	15	M	M	-
T39	70	M	-	+	9	M	W	-
T40	65	M	+++	-	12	L	M	-
T41	50	F	-	-	16	M	M	-
T42	45	M	++	+	NA	M	M	-
T43	60	F	-	-	27	M	P	-
T44	50	M	+++	+	9	M	M	-
T45	60	M	++	+	12	M	M	-
T46	65	M	-	-	9	L	M	-
T47	50	M	+++	-	9	L	M	-
T48	56	F	+++	-	18	M	P	-

T49	58	M	-	-	NA	L	M	-
T50	50	M	-	-	16	L	M	-
T51	50	M	+++	-	NA	L	M	-
T52	50	F	-	-	30	M	W	-
T53	60	M	+++	-	16	L	M	Exon 18, GGC to GAC G719D
T54	70	F	-	+	8	U	M	-
T55	60	M	+++	-	15	M	M	-

[1]Age in years [2]Sex:: M=male, F=female; [3]Tobacco use. Approximately 25g of tobacco is consumed while smoking water pipe once; - =non-smoker; +=1-5times smoking /day (25g-125g), ++=6-10 times smoking /day (150g-250g), +++=11-15 times smoking /day smoking (275g-375g); [4]Consumption of snuff: -= no, +=yes; [5]Salt tea consumed X 100 ml; [6]Site: U=upper one-third, M=middle one-third, L=lower one-third; [7]Grade: PD=poorly differentiated, MD=moderately differentiated, WD=well differentiated

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