

## Fibroblast growth factor receptor 3 S249C mutation in virus associated squamous cell carcinomas

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**Abstract.** An S249C mutation in fibroblast growth factor receptor 3 (*FGFR3*) gene was recently identified in patients with cervical carcinomas (CC). However, its importance in cervical tumorigenesis is still inconclusive. Apart from CC, nasopharyngeal carcinoma (NPC) is the other major virus associated squamous cell carcinoma. We sought to clarify the frequency of the *FGFR3* S249C mutation in 75 primary CC in the Thai population and to determine its prevalence in 69 primary NPC by PCR and restriction enzyme digestion. None of the patients but one NPC showed the enzyme digestion pattern consistent with the mutation. This is the first report demonstrating the role of *FGFR3* in the development of human NPC. This study confirms the low frequency of the *FGFR3* S249C mutation in CC. Nevertheless, the discovery of the mutation, not only in CC as reported by previous studies, but in NPC based on this report, suggests that *FGFR3* may play a significant role in human CC and NPC development.

### Introduction

Cervical and nasopharyngeal carcinomas are the two major virus associated squamous cell carcinomas. Cancer of the uterine cervix is one of the most common tumors affecting women worldwide with approximately 470,000 new cases diagnosed annually (1). Virtually all cervical carcinomas examined are positive for human papillomavirus (HPV) (2). Even though HPV is considered an essential cause of cervical cancer, it is certainly insufficient to induce transformation

and tumor progression (3). A long latency period between HPV infection and tumor appearance is suggested by the fact that the peak incidence of the disease is observed in females above 40 whereas HPV infection occurs in the 20s. Obviously, one important issue is to identify factors marking the transition of the HPV-containing cells to malignancy.

Recurrent genetic alterations in cervical cancer include losses of heterozygosity of many chromosomal regions, recurrent amplification of a few chromosomal sites, and microsatellite instability (3). Specific genes at these loci, however, still remain to be elucidated. Recently, a mutation, 746C→G (S249C), in a gene encoding fibroblast growth factor receptor 3 (*FGFR3*) was identified in 3 of 12 (25%) cervical carcinomas from the French population (4), making it the most common specific molecular genetic alteration in cervical cancer. However, two more recent articles analyzing a larger number of cervical carcinomas refuted the importance of the *FGFR3* activation in cervical tumorigenesis (5,6).

Nasopharyngeal carcinoma (NPC) is relatively common in South China and Southeast Asia with an incidence of 3 to 10 per 100,000 people/year compared to less than 1 per 100,000 people/year in most parts of the world (7,8). Epstein-Barr virus (EBV) appears to be an important etiological factor for NPC (9,10). Several recurrent genetic alterations in NPC have been identified including losses of heterozygosity of many chromosomal regions, recurrent amplification of a few chromosomal sites, and microsatellite instability (11,12). Because of the similarity between cervical cancer and NPC as to their ubiquity in Thailand, their virus associated tumorigenesis, and their histopathology, we sought to clarify the role of the *FGFR3* S249C mutation, the only mutation identified in the *FGFR3* gene in cervical cancers to date, in a large sample of cervical carcinomas in the Thai population and to determine its role in nasopharyngeal tumorigenesis.

### Materials and methods

Having obtained informed consent, slides of paraffin-embedded dissected tissues from 23 cervical carcinoma patients, collected between 1997 and 2000, were washed with xylene solution followed by 100%, 95% and 70%

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ethanol, respectively, resuspended in 1 ml lysis buffer (10% SDS, 0.75 M NaCl, 0.24 M EDTA pH 8.0, and 20 µg/ml proteinase K) and rotated overnight at 55°C. Equal volumes of phenol-chloroform were added to the solution and centrifuged at high speed for 15-20 min. DNA was precipitated with 3 M sodium acetate, washed twice with 100% and 70% ethanol, air dried, and resuspended in a volume of 50-200 µl dH<sub>2</sub>O.

Primary tissues were collected from 52 patients with cervical carcinoma and 69 patients with nasopharyngeal carcinoma. The tissues were divided into two pieces, the first part was sent for routine histological examination whereas the second part was incubated in 1.2 ml digestion buffer per 100 mg tissue on a shaker at 50°C overnight. This was followed by phenol-chloroform extraction and ethanol precipitation. The resulting pellets were air dried, and resuspended in dH<sub>2</sub>O.

The samples were PCR amplified by one of the following two methods. First, using nested PCR for amplification, the primers FR3F7: 5'-AGT GGC GGT GGT GGT GAG GGA GG-3' and FR3R7: 5'-AAT CCT TCA CGC AAC CCG CAG CCA-3' were used for the first round PCR reaction in total volume of 20 µl comprising 200 ng of each primer, 1.2 mmol/l MgCl<sub>2</sub>, 200 µmol/l dNTPs, 1 U Taq polymerase with the appropriate reaction buffer supplied by the manufacturer's, and 50 ng of genomic DNA. The reaction consisted of 30 cycles at 94°C for 30 sec, 64°C for 45 sec and 72°C for 1 min. A second nested PCR reaction was generated by using 1 µl of the first PCR product and the same reaction mix as above except for primers INF1: 5'-CTG AGC GTC ATC TGC CCC-3' and INR1: 5'-CGC CTG CAG GAT GGG CCC-3'. The nested reaction comprised 5 cycles at 94°C for 30 sec, 64°C for 45 sec and 72°C for 30 sec. These conditions result in amplification of a 55 bp product containing codon 249 in exon 7 of the *FGFR3* gene.

Secondly, the samples were amplified using the FR3F7 and INR1 primers in the 35-cycle reactions at 94°C for 45 sec, 64°C for 45 sec and 72°C for 1 min, from which an 87-bp PCR product containing codon 249 was obtained. We also designed a mutated positive control primer: 5'-CGC CTG CAG GAT GGG CCG GTG CGG GCA G-3' used as the reverse primer for amplification with either the forward primer INF1 applying the first method or the forward primer FR3F7 applying the second method. The PCR products obtained with the mutated primer are of the same size as with the normal primers but contain a nucleotide change at position 746 (746C→G) of codon 249, which serves as a restriction site for the enzyme, *Fnu4HI*. All PCR products were digested with *Fnu4HI* (New England Biolabs) overnight according to the manufacturer's protocol, electrophoresed through a 12% polyacrylamide gel and stained with ethidium bromide.

## Results

Of the 75 cervical carcinoma specimens, 55 (73.3%) were squamous cell and 16 (21.3%) were adenocarcinoma. A histology subtype was unavailable for four (5.3%) cases. As for FIGO staging, one was stage IA, 23 were stage IB, two were stage IIA, 15 were stage IIB, 28 were stage IIIB, one

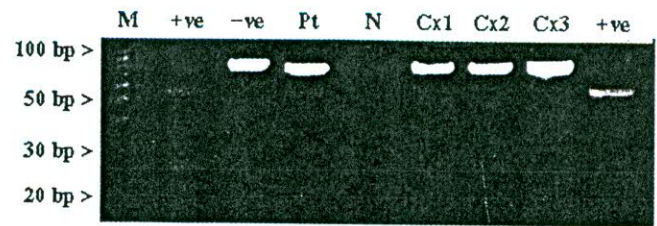


Figure 1. *Fnu4HI* digestion of PCR products. Lane 1 is a 10 bp marker. Lanes 2 and 9 are positive controls containing the *FGFR3* S249C mutation generated by amplification with the mutated reverse primer. Lane 3 is a negative control amplified from white blood cells of a normal person. Lane 4 is the patient #389 demonstrating the 60- and 27-bp bands consistent with the S249C mutant allele. Lane 5 is a control without DNA. Lanes 6-8 are samples obtained from three patients with cervical cancer.

was stage IVA, and two were stage IVB. No stage was available for three cases. None of the 75 samples had a *Fnu4HI* enzyme digestion pattern consistent with the *FGFR3* S249C mutation.

Surprisingly, applying the same method, we found one of the 69 primary nasopharyngeal cancer tissues displaying a pattern consistent with the *FGFR3* S249C mutation (Fig. 1). An 87-bp PCR product of the patient #389 amplified by FR3F7 and INF1 primers was cut into 67 and 20 bp fragments by the *Fnu4HI* restriction enzyme, identical to the pattern of the positive control. The reaction was repeated thrice yielding identical results.

Patient #389 was a 66-year-old Thai male patient. He presented to the hospital because of recurrent epistaxis for 1 year. A right upper jugular lymph node, 2 cm in diameter, was palpated. Sinuscopy revealed ulcers at the roof of the nasopharynx. A nasopharyngeal biopsy was performed and the histopathology revealed carcinomatous changes consistent with undifferentiated type nasopharyngeal cancer. Computerized tomography of the nasopharynx revealed a mass at the nasopharyngeal roof extending to the right parapharyngeal space with right sphenoidal sinusitis. Bone scan showed no metastasis. Pure tone audiogram revealed mild left sensorineural hearing loss and mild right conductive hearing loss. Complete blood counts, BUN, creatinine, plasma glucose, VDRL, erythrocyte sedimentation rate, HIV antibody, prothrombin time, partial thromboplastin time, and chest X-ray were within normal limits. Stage T<sub>2B</sub>N<sub>1</sub>M<sub>0</sub> was given. The patient was treated with radical radiation applying 6,700 cGy for 6.5 weeks. At his last visit two years after completion of the radiation treatment, he was still in clinical remission.

## Discussion

Fibroblast growth factors constitute a family of related mitogens with at least 18 members characterized to date (13). Four fibroblast growth factor receptors (FGFR1-4) make up a family of structurally related receptors encoded by four different genes (14). These receptors are composed of three extracellular immunoglobulin (Ig)-like domains, a transmembrane domain and a tyrosine kinase domain. The *FGFR3* gene on chromosome 4p16.3 consists of 19 exons spanning 16.5 kb (15,16). Specific point mutations in *FGFR3*

