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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_2O.

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_2O.

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 $MgCl_2$, 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

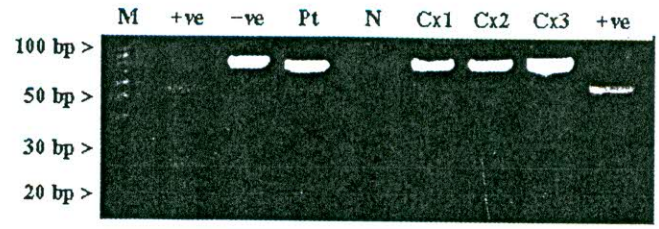

Figure 1. *Fnu4HI* digestion of PCR products. Lane 1 is a 10 bp marker. Lanes 2 and 9 are positive controls containing the *FGFR3* S249C mutation generated by amplification with the mutated reverse primer. Lane 3 is a negative control amplified from white blood cells of a normal person. Lane 4 is the 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 $T_{2B}N_1M_0$ 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*

were found associated with several autosomal dominant craniosynostoses and skeletal dysplasias including Muenke craniosynostosis, achondroplasia, hypochondroplasia, and thanatophoric dwarfism (13). Its oncogenic role was first proposed in multiple myeloma (17,18). After the chromosomal translocation t(4;14)(p16.3;q32), the *FGFR3* gene is translocated to the immunoglobulin heavy chain locus at chromosome 14q13. This mutation results in increased levels of *FGFR3* expression, presumably contributing to tumorigenesis of multiple myeloma. In addition, dysregulation of *FGFR3* was recently found in approximately 50% colorectal cancer patients (19). However, there are some human malignancies without evidence of *FGFR3* abnormalities such as human prostate (20) and gastric (21) cancers.

The role of *FGFR3* in tumorigenesis of cervical cancer was first elucidated by Cappellen *et al* (4). After having detected *FGFR3* expression in normal bladder and cervix epithelia, they screened for mutations by PCR-SSCP analysis of the whole coding region of *FGFR3* and found point mutations in 9 of 26 (35%) bladder carcinomas and 3 of 12 (25%) cervical carcinomas from the French population (4). All three cervical cancers had an S249C mutation and were somatic in nature. The identical germ line mutation causes thanotophoric dwarfism, a lethal form of skeletal dysplasia (22).

However, Yee *et al* (5) analyzed 104 primary cervical cancers from the urban Northeastern United States by direct sequencing of the 161-base-pair polymerase chain reaction product containing codon 249 and found only the wild-type sequence. Similarly, Wu *et al* (6) performed sequence-based mutational analysis of *FGFR3* in 51 primary cervical cancers from Columbia, Spain and the Philippines and 7 cervical carcinoma-derived cell lines. They analyzed exons 7, 10, 13, 15 and 19, which encompassed all the previously described *FGFR3* mutations, and found only one primary tumor with the S249C. Collectively, the S249C has been identified in only 4 of 157 primary cervical cancers and none of 7 cell lines. The mutation is somatic in nature.

We analyzed the S249C by PCR-restriction enzyme digestion of 75 primary cervical cancers and found that none of them contained the mutation. This finding reduces the prevalence of the *FGFR3* S249C mutation in primary cervical cancers to 4 in 232. It supports the findings of Yee *et al* (5) and Wu *et al* (6) that the *FGFR3* S249C is unlikely to represent a common mutation in cervical carcinoma. The discrepancy between the data of Cappellen *et al* (4) and our data could be due to different ethnicity or a number of environmental influences such as different HPV strains. Several HPV types have been found associated with this tumor, particularly types 16, 18, 33 and 42 (23). Different types could have different effects. Another possibility would be that there are other mutations affecting this gene in different populations and that with our methods we could only detect the S249C mutation. However, it is the only mutation observed in cervical cancers following a mutation screen of the entire *FGFR3* coding region by Cappellen *et al* (4) and a sequence analysis of all exons encompassing all *FGFR3* mutations previously described by Wu *et al* (6). We believe the results of Cappellen *et al* could have resulted from the small number of samples analyzed.

As there are several similarities between cervical cancers and nasopharyngeal cancers, we analyzed 69 primary nasopharyngeal cancers for the S249C mutation. One of these had the pattern of the *Fnu4HI* enzyme digestion identical to the positive control, which contains the *FGFR3* S249C mutation. Collectively, this patient is the only one out of 144 cervical and nasopharyngeal cancer patients we analyzed who displayed the pattern. As seen in Fig. 1, the undigested 87-bp band of the patient is much denser than the digested 27- and 60-bp bands throughout the three repetitive experiments. We believe this was due to the presence of other normal cells such as lymphocytes, which should contain only normal alleles and hence, not be digested by the enzyme in the specimen we biopsied and used for DNA extraction. Since the S249C is a known mutation and the patient #389 showed the unequivocal, repeatable and identical digestion pattern, we consider sequencing unnecessary. The nature of the mutation should be somatic, not germ line. Unfortunately, the blood sample from the patient is not available to confirm the hypothesis. Nevertheless, this is the first report to demonstrate that *FGFR3* may play a role in the development of human nasopharyngeal carcinoma.

In summary, even though its contribution may be minor, *FGFR3* S249C has been demonstrated to play a role in human cervical and nasopharyngeal oncogenesis.

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References

1. Cannistra SA and Niloff JM: Cancer of the uterine cervix. N Engl J Med 334: 1030-1038, 1996.
2. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ and Munoz N: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189: 12-19, 1999.
3. Lazo PA: The molecular genetics of cervical carcinoma. Br J Cancer 80: 2008-2018, 1999.
4. Cappellen D, De Oliveira C, Ricol D, de Median S, Bourdin J, Sastre-Garau X, Chopin D, Thiery JP and Radvanyi F: Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet 23: 18-20, 1999.
5. Yee CJ, Lin O and Boyd J: Analysis of fibroblast growth factor receptor 3 S249C mutation in cervical carcinoma. J Natl Cancer Inst 92: 1848-1849, 2000.
6. Wu R, Connolly D, Ngelangel C, Bosch FX, Munoz N and Cho KR: Somatic mutations of fibroblast growth factor receptor 3 (FGFR3) are uncommon in carcinomas of the uterine cervix. Oncogene 19: 5543-5546, 2000.
7. Vokes EE, Liebowitz DN and Weichselbaum RR: Nasopharyngeal carcinoma. Lancet 350: 1087-1091, 1997.
8. Her C: Nasopharyngeal cancer and the Southeast Asian patient. Am Fam Physician 63: 1776-1782, 2001.
9. Niedobitek G: Epstein-Barr virus infection in the pathogenesis of nasopharyngeal carcinoma. Mol Pathol 53: 248-254, 2000.

10. Shotelersuk K, Khorprasert C, Sakdikul S, Pornthanakasem W, Voravud N and Mutirangura A: Epstein-Barr virus DNA in serum/plasma as a tumor marker for nasopharyngeal cancer. Clin Cancer Res 6: 1046-1051, 2000.
11. Hui AB, Lo KW, Leung SF, Teo P, Fung MK, To KF, Wong N, Choi PH, Lee JC and Huang DP: Detection of recurrent chromosomal gains and losses in primary nasopharyngeal carcinoma by comparative genomic hybridisation. Int J Cancer 82: 498-503, 1999.
12. Mutirangura A, Tanunyutthawongese C, Pornthanakasem W, Kerekhanjanarong V, Sriuranpong V, Yenrudi S, Supiyaphun P and Voravud N: Genomic alterations in nasopharyngeal carcinoma: loss of heterozygosity and Epstein-Barr virus infection. Br J Cancer 76: 770-776, 1997.
13. Passos-Bueno MR, Wilcox WR, Jabs EW, Sertie AL, Alonso LG and Kitoh H: Clinical spectrum of fibroblast growth factor receptor mutations. Hum Mutat 14: 115-125, 1999.
14. Wilkie AO, Morriss-Kay GM, Jones EY and Heath JK: Functions of fibroblast growth factors and their receptors. Curr Biol 5: 500-507, 1995.
15. Perez-Castro AV, Wilson J and Altherr MR: Genomic organization of the human fibroblast growth factor receptor 3 (FGFR3) gene and comparative sequence analysis with the mouse Fgfr3 gene. Genomics 41: 10-16, 1997.
16. Wuchner C, Hilbert K, Zabel B and Winterpacht A: Human fibroblast growth factor receptor 3 gene (FGFR3): genomic sequence and primer set information for gene analysis. Hum Genet 100: 215-219, 1997.
17. Chesi M, Nardini E, Brents LA, Schrock E, Ried T, Kuehl WM and Bergsagel PL: Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nat Genet 16: 260-264, 1997.
18. Richelda R, Ronchetti D, Baldini L, Cro L, Viggiano L, Marzella R, Rocchi M, Otsuki T, Lombardi L, Maiolo AT and Neri A: A novel chromosomal translocation t(4; 14)(p16.3; q32) in multiple myeloma involves the fibroblast growth-factor receptor 3 gene. Blood 90: 4062-4070, 1997.
19. Jang JH, Shin KH, Park YJ, Lee RJ, McKeehan WL and Park JG: Novel transcripts of fibroblast growth factor receptor 3 reveal aberrant splicing and activation of cryptic splice sequences in colorectal cancer. Cancer Res 60: 4049-4052, 2000.
20. Naimi B, Latil A, Berthon P and Cussenot O: No evidence for fibroblast growth factor receptor 3 (FGFR-3) R248C/S249C mutations in human prostate cancer. Int J Cancer 87: 455-456, 2000.
21. Shin EY, Lee BH, Yang JH, Shin KS, Lee GK, Yun HY, Song YJ, Park SC and Kim EG: Up-regulation and co-expression of fibroblast growth factor receptors in human gastric cancer. J Cancer Res Clin Oncol 126: 519-528, 2000.
22. Tavormina PL, Shiang R, Thompson LM, Zhu YX, Wilkin DJ, Lachman RS, Wilcox WR, Rimoin DL, Cohn DH and Wasmuth JJ: Thanatophoric dysplasia (types I and II) caused by distinct mutations in fibroblast growth factor receptor 3. Nat Genet 9: 321-328, 1995.
23. Zur Hausen H and de Villiers EM: Human papillomaviruses. Annu Rev Microbiol 48: 427-447, 1994.