Lack of Fas-pathway Gene Mutations in Primary Resected Esophageal Squamous Cell Carcinoma

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Background: The Fas system regulates a number of physiological and pathological processes of cell death. Many types of cancer cells are resistant to Fas-mediated apoptosis by several mechanisms, including mutations of the genes involved in Fas-mediated apoptosis. The present study aimed to detect Fas-pathway gene alterations in primary resected esophageal squamous cell carcinoma (ESCC).

Methods: Immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) were used to analyze the expression of Fas in 34 archival specimens from patients with primary resected ESCC. We then explored the possibility of mutations in the genes involved in the proximal pathway of Fas-mediated apoptosis (Fas, FADD). We analyzed exon 9 (death domain) of the Fas gene and exon 1 (death effective domain) of the FADD gene for gene mutations in these 34 specimens by polymerase chain reaction and DNA sequencing.

Results: Immunohistochemistry and RT-PCR revealed that Fas was expressed in all primary resected ESCC specimens analyzed. However, we did not detect any structural alterations in exon 9 of the Fas gene or exon 1 of the FADD gene.

Conclusions: Inhibition of Fas-mediated apoptosis may not be due to gene mutations in the critical exon 9 of the Fas gene and exon 1 of the FADD gene in esophageal squamous cell carcinoma.

Key words: Fas, apoptosis, esophageal squamous cell carcinoma

Apoptosis (programmed cell death) is considered an important homeostatic mechanism that balances cell production with cell death and maintains correct cell numbers in the body in physiological and pathological conditions. Aberration in regulation of apoptosis is expected to contribute to tumor progression and oncogenesis, and cancer cells gain selective growth advantages by blocking apoptosis. The Fas system has been recognized as a major pathway for the induction of apoptosis in cells and tissues. Fas is a member of the death receptor subfamily of the tumor necrosis factor receptor superfamily. Fas has three cysteine-rich extracellular domains and an intracellular death domain essential for signaling.
Ligation of Fas by either an agonistic antibody or by its natural ligand transmits a “death signal” to the target cells, potentially triggering apoptosis.\(^3,\)\(^4\) Stimulation of Fas results in aggregation of its intracellular death domain (DD), leading to the recruitment of two key signaling proteins that together with the receptor form the death-inducing signaling complex (DISC). Fas-associated death domain (FADD)/MORT-1 couples through its C-terminal DD to cross-linked Fas receptors and recruits caspase 8 through its N-terminal death effector domain (DED) to the DISC.\(^5,\)\(^6\) The oligomerization of caspase 8 may result in self-activation of proteolytic activity and trigger the interleukin-1 beta-converting enzyme protease cascade and induce apoptosis.

Fas is widely expressed in normal and neoplastic cells,\(^7\) but the expression of this protein does not necessarily predict susceptibility to killing.\(^8\) Fas protein is constitutively expressed in normal esophageal epithelium, but expression is frequently less and sometimes undetectable in esophageal carcinomas.\(^8\) Yoshinaga et al demonstrated that Fas resistance in transfected human esophageal carcinoma cell lines is related to tumor progression in vitro and in vivo.\(^9\) This can reflect the presence of inhibiting mechanisms of Fas-mediated apoptosis in esophageal carcinoma. Fas-mediated apoptosis can be blocked by several mechanisms, including production of soluble Fas,\(^10\) lack of cell-surface Fas expression,\(^11\) overexpression of inhibitory proteins in signal transduction pathways such as Fas associated phosphatase-1 and FLICE-like interleukin protein,\(^12,\)\(^16\) and alterations in the genes involved in this pathway.

The key role of the Fas system in negative growth regulation has been studied mostly within the immune system, and the mutations of the Fas gene in cancer patients have been described solely in lymphoid-lineage malignancies, including multiple myeloma, adult T-cell leukemias, childhood T-cell lymphoblastic leukemias, non-Hodgkin's lymphomas, and diffuse large B-cell lymphoma.\(^12-16\) In these lymphoid-lineage malignancies, resistance against Fas-mediated apoptosis may lead to a longer survival of affected tumor cells and might contribute to tumorigenesis. There is mounting evidence that disruption of the Fas system frequently occurs in non-lymphoid malignancies as well.\(^17-19\) To date, however, somatic mutations of the Fas-pathway gene, one of the possible mechanisms that mediate the disruption of the Fas system, have not yet been reported in esophageal malignancies.

It is well known that the location of a mutation domain is important in predicting the function of mutants. The DD in the Fas is evolutionarily highly conserved and is shown to be necessary and sufficient for the transduction of an apoptotic signal.\(^1\) Also, most of the somatic mutations of Fas detected in previous reports are located in the DD.\(^19\) The DED in FADD is essential for the recruitment of caspase 8 to activate Fas signaling.\(^15\) Most of the somatic mutations of FADD detected in a previous report are also located in the DED.\(^19\) In the present study, in order to characterize the potential apoptosis-resistant pathway of the Fas system in human esophageal squamous cell carcinoma, we analyzed a series of 34 esophageal squamous cell carcinomas for detection of somatic mutations of the DD of the Fas gene and the DED of the FADD gene.

**METHODS**

**Patients and tissue samples**

Tumor samples were obtained from 34 consecutive patients who underwent surgical resection for esophageal squamous cell carcinoma. No patients had received chemotherapy or radiotherapy before the operation. The tumors were graded according to criteria recommended by the WHO and staged according to the tumor-node-metastasis classification. They consisted of 2 stage I, 14 stage II, 14 stage III and 4 stage IV carcinomas.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis of Fas mRNA expression**

Total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX, U.S.A.) from the 34 primary esophageal tumors. RNA 2 ug was reversely transcribed using the cDNA Cycle Kit (Invitrogen, Carlsbad, CA, U.S.A.). Amplification of cDNA was performed using primers specific for Fas and β-actin (internal control). Primers specific for Fas cDNA amplification were: (F)5'-gaccgcaataacagtgcagta and (R)5'-ctgtttcaggattgaaggttggagatt. Primers specific for β-actin cDNA amplification were: (F)5'-gtggggcgcagctggagctta and (R)5'-ctgtttcaggattaagttggagatt. PCR amplification for Fas and β-actin mRNA was performed under the following conditions to
yield 488 and 400 bp products, respectively: initial denaturation at 94°C, 3 min; 25 cycles at 94°C, 1 min; 60°C, 2 min; 72°C, 2 min; and a final extension of 72°C, 4 min. The PCR products were resolved on 1.2% agarose gel, stained with ethidium bromide and visualized by UV illumination.

**Immunohistochemistry**

Paraffin-embedded tumor specimens that had been fixed in neutral buffered formalin were sectioned (5 µm) and placed on poly-L-lysine-coated glass slides. After microwave pretreatment in 10 mM citric acid monohydrate (pH 6.0) three times for 5 min at 750 W, slides were incubated for two hours at 4°C with monoclonal antibodies against Fas (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After a second incubation with biotin-conjugated anti-mouse antibody, slides were incubated with avidin-biotin-peroxidase reagent (LSAB kit, Dakopatts, Carpinteria, CA 93013, U.S.A.). Reaction products were visualized by immersing slides in diaminobenzidine tetrachloride and finally counterstained with hematoxylin. In the negative controls, the primary antibodies were replaced by dilution buffer. Positive staining of normal squamous cell epithelium provided a positive internal control for immunostaining.

Using light microscopy, the immunohistochemical expression of Fas was examined by two observers without knowledge of the clinical outcome.

**Direct sequencing**

Genomic DNA from 34 primary esophageal tumor samples was amplified by PCR using primers Fas9F (5’-ggttttcactaatgggaatttcat-3’) and Fas9R (5’-gaatttgttgttttcactcta-3’) (PCR product: 421 bp) for Fas exon 9 and primers FADDex1F (5’-ccgcgccttgacaccgcc-3’) and FADDex1R (5’-ccaacccttaacgccggaggacca-3’) (PCR product: 365 bp) for FADD exon 1. (Eurofins MWG Operon, Ebersberg, Germany). Cycle sequencing of the purified PCR product was carried out with one of the PCR primers using the Big-dye terminator sequencing kit (Applied Biosyste, Foster city, CA 94404, U.S.A.). The Sephadex G-50-purified cycle sequencing products were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA 94404, U.S.A.).

**RESULTS**

A total of 34 samples of primary resected esophageal squamous cell carcinoma were processed for Fas expression by immunohistochemistry and RT-PCR. The Table 1 lists the clinicopathological features of all patients.

**Immunohistochemistry of Fas expression in primary resected ESCCs**

The Fas protein in primary resected ESCC was assessed by immunohistochemical stains. It was found to be constitutively expressed in normal esophageal mucosa, predominantly in the intermediate and outer layers of normal esophageal mucosa. Down-regulation of Fas expression was observed with the advancement of the disease process, as described by Gratas et al.(8) Fas expression was diffuse in early stage, low grade tumor cells, as shown in Fig. 1, but not in the stroma.

<table>
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RT-PCR for Fas mRNA expression in primary resected ESCCs

The Fas mRNA in primary resected ESCC was assessed by RT-PCR. As shown in Fig. 2, a 488 bp product was found in all esophageal tumor tissues, corresponding to the expected Fas mRNA.

Direct sequencing for Fas-pathway gene analysis

Genomic DNA was isolated and analyzed for potential mutations in Fas exon 9 and FADD exon 1 using direct sequencing of PCR products. Fas exon 9 is coding for the death domain, while the FADD exon is coding for the death effector domain. In the present study, selection of these two exons was based on the results from previous studies, where mutations were detected almost exclusively in these exons. Nevertheless, in our analysis, none of the esophageal squamous cell carcinoma samples tested showed evidence of mutations in Fas exon 9 and FADD exon 1 by direct sequencing.

DISCUSSION

In the present study, we identified no mutations in exon 9 of the Fas gene or exon 1 in the FADD gene, coding for the critical death and death effector domains of the Fas death pathway, respectively. Structural alterations in Fas pathway genes may constitute one of the putative defects in the Fas signaling pathway. Mutations of Fas pathway genes in cancer patients were first described in lymphoid lineage malignancies. Recently, evidence has been report-
ed that Fas pathway gene mutations may also occur in non-lymphoid malignancies, such as non-small cell lung cancer and transitional cell carcinoma of the bladder.\textsuperscript{19-22} The mutations found were located mainly in exon 9 of the \textit{Fas} gene and exon 1 of the \textit{FADD} gene. These mutations in the death domain of \textit{Fas} and death effector domain of \textit{FADD} affect the phenotype in a dominant negative fashion owing to interference with recruitment of the adaptor protein FADD and procaspase-8 to the death domain.

In this study, we could not detect any alteration in the primary structure of \textit{Fas} exon 9 and \textit{FADD} exon 1 in esophageal squamous cell carcinomas by direct sequencing. This is not a totally unexpected finding since, except for bladder cancer, a rather low percentage of Fas pathway gene mutations have been detected in most tumors tested.\textsuperscript{22} Moreover, recent reports have not shown structural alterations in \textit{Fas} in colorectal cancer, squamous cell carcinoma of the skin or breast cancer.\textsuperscript{23-25} We could conclude that Fas pathway gene mutations might not be a main mechanism of disruption of the Fas death system in esophageal squamous cell carcinomas.

\textit{Fas} expression is a useful independent predictor of prognosis in esophageal squamous cell carcinomas as shown in a previous report and in our own study (unpublished).\textsuperscript{26} The question remains why Fas protein expression is subnormal in a high proportion of esophageal squamous cell carcinomas. Although Fas down-regulation has the capacity to reduce cellular responses to lethal stimuli, it is significant that mutational inactivation of this gene is not observed in esophageal squamous cell carcinomas. Besides \textit{Fas} gene mutations, a number of other mechanisms that may lead to resistance in Fas-mediated apoptosis have been proposed and are currently under investigation. We are also investigating the promoter and epigenetic modification of \textit{Fas}.

It is well known that the location of a mutation domain is important in predicting the function of mutants. The DD in the \textit{Fas} is evolutionarily highly conserved and is shown to be necessary and sufficient for the transduction of an apoptotic signal.\textsuperscript{(1)} Also, most somatic mutations of \textit{Fas} detected in previous reports are located in the DD.\textsuperscript{19} The DED in \textit{FADD} is essential for the recruitment of caspase 8 to activate Fas signaling.\textsuperscript{27} Most of the somatic mutations of \textit{FADD} detected in Shin et al’s report were also located in the DED.\textsuperscript{19} So we focused our study on detection of somatic mutants in the DD of \textit{Fas} and the DED of \textit{FADD}.

We conclude that inhibition of the Fas pathway in esophageal squamous cell carcinomas is not due to gene mutations in \textit{Fas} gene exon 9 and \textit{FADD} gene exon 1. Further investigation is necessary to explore the mechanisms of resistance to Fas mediated apoptosis in esophageal squamous cell carcinomas.

**REFERENCES**

14. Beltinger C, Kurz E, Bohler T. CD95 (APO-1/Fas) mutations in childhood T-lineage acute lymphoblastic
手術切除的食道鱗狀上皮癌中並無 Fas 途徑的基因突變

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背 景：Fas 系統調節許多與細胞死亡相關的生理及病理過程。許多不同的癌細胞曾經由幾種機制，包括 Fas 系統的基因突變來對抗 Fas 系統引起的細胞計畫死亡。這個研究即是要偵測手術切除的食道鱗狀上皮癌中是否有 Fas 途徑的基因改變。

方 法：三十四個接受手術切除的食道鱗狀上皮癌病患的癌組織檢體，利用免疫組織染色及反轉錄酶-聚合酶鍵反應去分析 Fas 的表現。同時也使用聚合酶反應及去氧核醣核酸定序去分析 Fas 基因的第九表現子 (死亡領域) 及 FADD 基因的第一表現子 (死亡有效領域) 中是否有基因突變的情形。

結 果：免疫組織染色及反轉錄酶-聚合酶鍵反應的研究顯示所有的食道癌檢體都有 Fas 的表現。但是我們無法在 Fas 基因的第九表現子及 FADD 基因的第一表現子中偵測到任何的基因突變。

結 論：抑制食道鱗狀上皮癌中 Fas 所引起的細胞計畫死亡的機轉可能與 Fas 基因第九表現子及 FADD 基因第一表現子的基因突變無關。

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關鍵詞：Fas，細胞計畫死亡，食道鱗狀上皮癌