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

Chih-Ling Ko, MD; Chiou-Ying Yang[2], PhD; Cheng-Yen Chung, MD;
Mei-Chin Wen[1], MD; Chung-Ping Hsu[1,5], MD; Jen Miaw, MD; Jiun-Yi Hsia[1,3,4], MD

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.
(Chang Gung Med J 2010;33:145-51)

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.[1] 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.[1] 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.[2]

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 intra-cellular death domain (DD), leading to the recruit-ment of two key signaling proteins that together with the receptor form the death-inducing signaling com-plex (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 neoplas-tic cells,[3] but the expression of this protein does not necessarily predict susceptibility to killing.[7] Fas pro-tein 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 mecha-nisms of Fas-mediated apoptosis in esophageal carci-noma. Fas-mediated apoptosis can be blocked by several mechanisms, including production of soluble Fas,[10] lack of cell-surface Fas expression,[11] overex-pression of inhibitory proteins in signal transduction pathways such as Fas associated phosphatase-1 and FLICE-like interleukin protein,[5,6] 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 lym-phoid-lineage malignancies, including multiple myeloma, adult T-cell leukemias, childhood T-cell lymphoblastic leukemias, non-Hodgkin's lym-phomas, 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 suffi-cient 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 cas-pase 8 to activate Fas signaling.[1] Most of the somat-ic 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-resis-tant 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 consecu-tive 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 classifica-tion. They consisted of 2 stage I, 14 stage II, 14 stage III and 4 stage IV carcinomas.

Reverse transcription-polymerase chain reac-tion (RT-PCR) analysis of Fas mRNA expres-sion

Total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX, U.S.A.) from the 34 prima-ry esophageal tumors. RNA 2 ug was reversely tran-scribed 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'-gacccagaataccaagtgcagatg-ta and (R)5'-ctgtttcaggatttaaggttggagatt. Primers spe-cific for β-actin cDNA amplification were: (F)5'-gtggggcgccccaggcacc and (R)5'-ctccttaatgtcacgcac-gatttc. 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 94C, 3 min; 25 cycles at 94C, 1 min; 60C, 2 min; 72C, 2 min; and a final extension of 72C, 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 diaminobenidine 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 Fasex9F (5'-ggttttcactaatgggaatttcat-3') and Fasex9R (5'-gaatttgttgtttttcactcta-3') (PCR product: 421 bp) for *Fas* exon 9 and primers FADDex1F (5'-ccg-gccgcttgcagaccc-3') and FADDex1R (5'-ccaacc-taacagcctggcgacca-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, Fostercity, 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 1. Clinicopathological Features of 34 ESCCs

Variable	
Sex	
Male	34
Female	0
Age	
≤ 60	19
> 60	15
Stage (UICC)	
I & II	16
III & IV	18
Histological differentiation	
Well	2
Moderate	19
Poor	13
Lymph node metastasis	
Positive	18
Negative	16
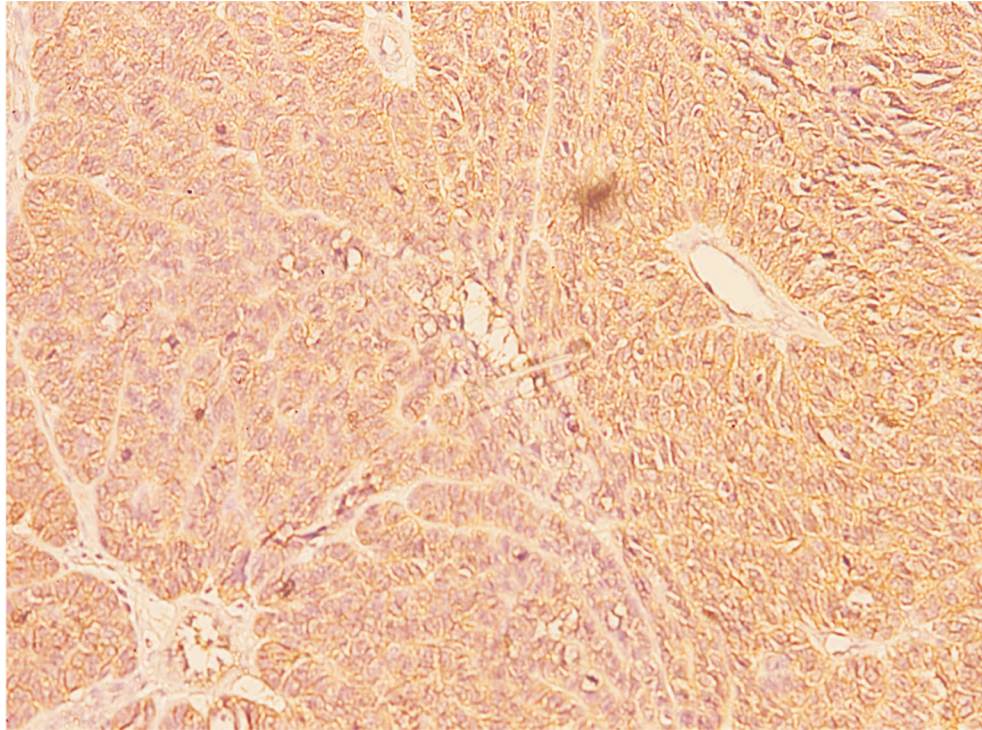
Fig. 1 Immunohistochemical staining of Fas (x 200) in primary resected ESCC. Fas expression was diffuse in the cytoplasm of tumor cells.

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.
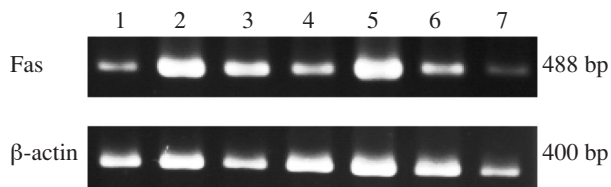
Direct sequencing for Fas-pathway gene analysis

Genomic DNA was isolated and analyzed for potential mutations in *Fas* exon 9 and *FADD* exon 1

Fig. 2 Fas expression in ESCC detected by RT-PCR. Expression of β-actin served as a positive control. All seven patients with ESCCs expressed Fas.

using direct sequencing of PCR products. *Fas* exon 9 is coding for the death domain, while the *FADD* exon is coding for the death effecter 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 effecter 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.[11] 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.[19-22] The mutations found were located mainly in exon 9 of the *Fas* gene and exon 1 of the *FADD* gene. These mutations in the death domain of *Fas* and death effector domain of *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 *Fas* exon 9 and *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.[22] Moreover, recent reports have not shown structural alterations in *Fas* in colorectal cancer, squamous cell carcinoma of the skin or breast cancer.[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.

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).[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 *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 *Fas*.

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 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.[1] Most of the somatic mutations of *FADD* detected in Shin et al's report were also located in the DED.[19] So we focused our study

on detection of somatic mutants in the DD of *Fas* and the DED of *FADD*.

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

REFERENCES

1. Nagata S. Fas-induced apoptosis. Cell 1997;88:355-65.
2. Itoh N, Nagata S. A novel protein domain required for apoptosis. Mutational analysis of human Fas gene. J Biol Chem 1993;268:10932-7.
3. Leithäuser F, Dhein J, Mechtersheimer G. Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. Lab Invest 1993;69:415-29.
4. Suda T, Nagata S. Purification and characterization of the Fas-ligand that induces apoptosis. J Exp Med 1994;179:873-9.
5. Sato T, Irie S, Kituda S, Reed JC. FAP-1: a protein tyrosine phosphatase that associates with Fas. Science 1995;268:411-5.
6. Irmler M, Thome M, Hahne M. Inhibition of death receptor signals by cellular FLIP. Nature 1997;388:190-5.
7. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993;75:1169-78.
8. Gratas C, Tohma Y, Barnas C. Up-regulation of Fas (APO-1/CD95) ligand and down regulation of Fas expression in human esophageal cancer. Cancer Res 1998;58:2057-63.
9. Yoshinaga K, Yamashita K, Mimori K, Tanaka F, Inoue H, Mori M. Activin a causes cancer cell aggressiveness in esophageal squamous cell carcinoma cells. Ann Surg Oncol 2008;15:96-103.
10. Natoli G, Janni A, Costanzo A. Resistance to Fas-mediated apoptosis in human hepatoma cells. Oncogene 1995;11:1157-64.
11. Bennett M, Macdonald K, Chan SW. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 1998;282:290-3.
12. Landowsky TH, Qu N, Buyuksal I. Mutations in the Fas antigen in patients with multiple myeloma. Blood 1997;90:4266-70.
13. Tamiya S, Etoh KI, Suzushima H. Mutations of CD95 (Fas/Apo1) gene in adult T-cell leukemia cells. Blood 1998;91:3935-42.
14. Beltinger C, Kurz E, Bohler T. CD95 (APO-1/Fas) mutations in childhood T-lineage acute lymphoblastic

leukemia. Blood 1998;91:3943-51.

15. Gaonbæck K, Straten PT, Ralfkiaer E. Somatic Fas mutations in non-Hodgkin's lymphoma: association with extranodal disease and autoimmunity. Blood 1998;92:3018-24.

16. Takahashi H, Feuerhake F, Kutok JL, Mont S, Dul-Cin P, Neuberg D. Fas death domain deletions and cellular FADD-like interleukin 1 beta converting enzyme inhibitory protein (long) overexpression: alternative mechanisms for deregulating the extrinsic apoptotic pathway in diffuse large B-cell lymphoma subtypes. Clin Cancer Res 2006;12:3265-71.

17. Nambu Y, Hughes SJ, Rchemtula A. Lack of cell surface Fas/APO-1 expression in pulmonary adenocarcinomas. J Clin Invest 1998;101:1102-10.

18. Ungefroren H, Voss M, Jansen M. Human pancreatic adenocarcinomas express Fas and Fas ligand yet are resistant to Fas-mediated apoptosis. Cancer Res 1998;58:1741-9.

19. Shin MS, Kim HS, Lee SH. Alterations of Fas-pathway genes associated with nodal metastasis in non-small cell lung cancer. Oncogene 2002;21:4129-36.

20. Yang Q, Zhu RQ, Fang Z, Xia D, Diao LM, Lin MQ. Expression and mutation of Fas-associated death domain (FADD) gene in non-small cell lung cancer. Ai Zheng 2005;24:321-6.

21. Vaishnaw AK, Orlinkck JR, Chu JL, Krammer PH, Chao MV, Elkon KB. The molecular basis for apoptotic defects in patients with CD95 (Fas/Apo-1) mutations J Clin Invest 1999;103:355-63.

22. Lee SH, Shin MS, Park WS, Kim SY, Dong SM, Pi JH, Lee HK. Alterations of Fas (APO-1/CD95) gene in transitional cell carcinomas of urinary bladder. Cancer Res 1999;59:3068-72.

23. Abdel-Rahman W, Arends M, Morris R. Death pathway gene Fas (Apo-1/CD95) and Bik (Nbk) show no mutations in colorectal carcinomas. Cell Death Differ 1999;6:387-8.

24. Lee SH, Shin MS, Kim HS. Somatic mutations of Fas (APO-1/CD95) gene in cutaneous squamous cell carcinoma arising from a burn scar. J Invest Dermatol 2000;114:122-6.

25. Mueschen M, Re D, Betz B. Resistance to CD95-mediated apoptosis in breast cancer is not due to somatic mutation of the CD95 gene. Int J Cancer 2001;92:309-10.

26. Shibakita M, Tachibana M, Dhar DK. Prognostic significance of Fas and Fas ligand expression in human esophageal cancer. Clin Can Res 1999;5:2464-9.

手術切除的食道鱗狀上皮癌中並無 Fas 途徑的基因突變

柯志霖　楊秋英[1]　莊政諺　文美卿[2]　繆珍　夏君毅[1,3,4]　徐中平[1,5]

背　景： Fas 系統調節許多與細胞死亡相關的生理及病理過程。許多不同的癌細胞會經由幾種機轉，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-聚合酶鏈反應去分析 Fas 的表現。同時也使用聚合鏈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： 免疫組織染色及轉錄酶-聚合酶鏈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。
(長庚醫誌 2010;33:145-51)

關鍵詞： Fas，細胞計畫死亡，食道鱗狀上皮癌