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Original article

Impact of cell death pathway genes Fas 21377AA and FasL 2844CC polymorphisms on the risk of developing non-small cell lung cancer

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ABSTRACT

Background: The signalling pathway Fas and FasL system plays a fundamental role in the regulation of apoptotic cell death and any disturbance of this pathway has been shown to promote immune escape and tumorigenesis. Many types of cancers show reduced expression of FAS and elevated FasL expression. The Fas21377G/A, and FasL2844T/C polymorphisms might be associated with increased risk of lung cancer.
Objective: The interplay between genetic polymorphisms could participate in cancer development. This study aimed to examine the contribution of Fas21377AA and FasL2844CC genotypes to risk of developing lung cancer.
Subjects and methods: A case-control study was conducted on 20 non small cell lung cancer (NSCLC) cases and 40 controls. Genotyping of Fas 21377AA and FasL 2844CC Single nucleotide polymorphisms (SNPs) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) were done to all subjects.
Results: The distribution of Fas and FasL genotypes showed a higher frequency of Fas AA genotype among patients compared to controls with an increased risk of lung cancer (OR 5.28; CI:1.35–20.65, P value .01). No statistically significant difference was found between patients and controls groups in respect to FasL genotypes. Gene to gene interaction between Fas and FasL genotypes showed significant differences between the patients and controls groups. As regards the combination between FasL TT+CT & Fas AA, FasL CC & Fas GG+GA and FasL CC & Fas AA genotypes where patients carrying FasL CC or Fas AA genotypes have increased risk to develop lung cancer, (OR 10.28, 95% CI; 1.68–62.74, P value .01), (OR 72, 95% CI; 5.55–132.99, P value .001) and (OR 9, 95% CI; 1.5–53.86, P value .01) respectively. The FasL-CC genotype showed 2.25 folds increased risk to develop lung cancer in non-smoker patients, P = .008. No correlation was found between the pathological types, the stage of lung cancer and the Fas and FasL genotypes.
Conclusion: The interaction of the cell death pathway genes Fas and FasL polymorphisms could be associated with the risk of lung cancer, in the same respect Fas AA genotype could also potentiate this risk.

1. Introduction

Lung cancer is claimed to be one of the leading causes of cancer related deaths over the world [1]. Since 1985, it has been the most common cancer diagnosed each year [2]. Respiratory system primary malignant tumors represent the fifth common tumors in the Egyptian registry material, being about 5.9% of the total malignancies [3].

Tumour evolution is a complex process with many interplaying mechanisms and factors, among these is apoptosis (programmed cell death). Disabling of apoptotic responses might be a crucial contributor not only of tumour cell survival but also the resistance to the anticancer therapy. Initiation of apoptosis is mainly dependant on the Fas–Fas ligand (FasL) pathway [4]. Any disequilibrium in the genes regulating this cell death pathway might lead to cancer formation and development eg. single nucleotide polymorphisms (SNPs) [5].

The Fas/FasL has been shown to influence cancer development through two opposite effects where Fas expression with functional signalling pathway trigger Fas mediated tumour cell killing by effector cells, meanwhile FasL expression on tumour cells could repel specific antitumour immune response turning tumour into an immune privileged sites [6].

The genes of Fas & FasL are located on chromosome 1 (10q24.1 and regions 23, respectively). Fas gene encodes for transmembrane protein I (319 amino acid residues) while FasL encodes for

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transmembrane protein II (281 amino acid residues) [7,8]. In addition to the proappoptotic property of Fas/FasL signaling pathway, several evidences demonstrated that this pathway can also activate many nonappoptotic pathways whose activation result in increased tumourigenicity. Decreased expression of Fas and/or increased expression of FasL are announced to be allied with different malignancies and may also favor malignant conversion and progression [5].

The Fas and FasL gene mutations that attenuated this apoptotic signal transduction have been declared to display an augmented threat to many cancer diseases. Numerous studies have declared the linkage between Fas/FasL polymorphisms and risk of breast cancer [9,10], gastric cancer [11,12], cervical cancer [13,14], esophageal cancer [15,16] and lung cancer [17,18]. Moreover, the impact of Fas and FasL polymorphisms on the increased risk of oesophageal squamous cell carcinoma (SCC) displayed a multiplicative gene–gene interaction and appeared to be associate with tobacco smoking [16]. Among candidate lung tumour progression (LTP) genes, FasL was claimed to be the most important gene whose expression changes may seriously influence lung tumour progression in mice [19]. Furthermore, the Fas/FasL system may have a considerable role in cancer beginning and prognosis. Therefore, SNPs which acquire the potential to change the expression of these genes have been anticipated to be crucial in the genetic propensity to cancer [4].

2. Objective

This work is an attempt to find an answer to a question why not all subjects exposed to tobacco smoking or indoor air pollution develop lung cancer? A search for genetic predisposition implicated in the development of lung cancer have been created to find the contribution of Fas 2377AA and FasL 2844CC polymorphisms to the risk of lung cancer.

2.1. DNA extraction

Genomic DNA was extracted from blood samples obtained from 40 controls and 20 lung cancer patients using QIAamp DNA extraction kit (Qiagen Hilden, Germany, Cat No. 51304) according to the manufacturer's instructions.

2.2. Genotyping of Fas 21377AA and FasL 2844CC SNPs by PCR-RFLP

All patients and controls were genotyped using PCR based restriction fragment length polymorphism (PCR-RFLP) methods as described previously [15]. The PCR primers for amplification of the Fas promoter region containing 21377G/A were 59-TGTGTGCACAAGGCTGGCGC and 59-TGCATCTGTCACTGCACTT ACCACCA, which produce a 122 bp fragment. In order to induce a restriction endonuclease site, we changed the 39 end of the reverse primer from CAC to CGC, which created a BstuI cutting site. For amplification of the FasL promoter region containing 2844T/C site, we used the primer pair of 59-CAGCTACTCGGAGGCCAAG and 59-GCTCTGAGGGGAGAGACCAT, which generates a 401 bp fragment. Amplification of these two DNA fragments was accomplished separately under the same conditions, in a 25 ml reaction mixture consisting of, 100 ng template DNA, 0.5 mmol/l each primer, 0.2 mmol/l dNTP, 2.0 mmol/l MgCl2, and 1.0 U of Taq DNA polymerase with 16 reaction buffer (Promega, Madison, WI, USA). The reaction was carried out in the following conditions: an initial melting step of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 45 s at 72 °C, and a final elongation step of 7 min at 72 °C. The restriction enzymes BstuI and BsrDI (New England Biolabs Inc., Beverly, MA, USA) were used to distinguish the Fas 21377G/A and FasL 2844T/C polymorphisms, respectively. The restriction products were separated on 2.5% agarose gel with ethidium bromide. The RFLPs of the two polymorphisms were readily discerned. After digestion with BstuI, the Fas 21377G allele generated 104 bp and 18 bp fragments whereas the variant 21377A allele generated a single 122 bp fragment. The FasL 2844C allele had a BsrDI restriction site that resulted in two bands (233 bp and 168 bp), and the 2844T allele lacked the BsrDI restriction site, producing a single 401 bp band [17].

2.3. Statistical analysis

All analyses were done using SPSS© Statistics version 17 ((SPSS Inc., Chicago, IL). Pearson's x2 test was used to compare differences in Categorical variables between patients and controls. The associations between the polymorphisms and risk of increasing lung cancer were expected by odds ratios (ORs) and their 95% confidence intervals (CIs), which were measured by logistic regression. Light and heavy smokers were classified by median pack year value of $\geq$20 for heavy smokers. All ORs were adjusted for age, sex, and smoking status or pack years, as possible. All statistical tests were two sided tests. The null hypotheses of additive and multiplicative gene–gene interaction was studied, then we assessed the desertions from additive and multiplicative interaction models by counting main effect variables and their outcome terms in the logistic regression model.

3. Subjects and methods

The work was a collaboration between National Cancer Institute (NCI) and National Research Center (NRC), Cairo, Egypt. A case–control study was conducted on 20 unrelated adult patients with primary lung cancer and 20 smokers and 20 non-smokers unrelated controls. All subjects included in the study were interviewed to fill a medical questionnaire with special consideration to the lifetime history of tobacco use, residence, occupational history and family history of cancer. Thorough clinical examination and chest radiography were applied. Blood sample was obtained from each subject for Genotyping of Fas 21377AA and FasL 2844CC SNPs by PCR-RFLP. Sample for histopathology examination of cancer was obtained from each patient either by open biopsy or via bronchoscopy. The exclusion criteria included previous history of cancer, metastasized cancer from other organs, patients with pulmonary fibrosis, acute interstitial pneumonia and previous radiotherapy or chemotherapy or receiving any anti-cancer treatment before enrollment in the study. The study was approved by the ethics committee of the National Research Center. The work has been carried out in accordance with the code of Ethics of the World Medical association (Declaration of Helsinki) for experiments in humans. All subjects were aware by the nature of the study and gave a written informed consent.

4. Results

The distribution of patients and controls characteristics were demonstrated in Table 1 and showed that both groups were matched as regards their sex. There was a statistically significant difference between the two groups in concern to the number of the smoked packs per year where all the smoking patients take more than 20 packs per year (P value < .05).

The distribution of Fas and FasL genotypes frequencies among patients and controls and their associations with the risk of lung cancer were demonstrated in Table 2 and showed that the frequency of Fas AA genotype was higher among the patient group in comparison to the control group and those carriers of this

mutant allele have increased risk of lung cancer (OR 5.28; CI: 1.35–20.65, P value .01). Upon adjustment of this comparison as regards the sex, smoking history and the smoked packs per year, both Fas AA & GA genotype frequencies demonstrated statistically significant differences between the two groups and the carriers of these genotypes have increased risk of lung cancer (OR 10; CI: 2.0–50.23, P value .005) & (OR 41.48; CI: 1.04–164.1, P value .04) respectively. On the other hand no statistically significant differences between patient and control groups in respect to FasL genotypes.

The combined effect of Fas and FasL genotypes on the risk of the lung cancer among patients and controls was demonstrated in Table 3 and showed significant differences between the patient and control groups as regards the combination between FasL TT +CT & Fas AA, FasL CC & FAS GG+GA and FasL CC & Fas AA genotypes where patients carrying either FasL CC or Fas AA genotypes have increased risk to develop lung cancer (OR 10.28, 95% CI; 1.68–62.74, P value .01), (OR 72, 95% CI; 5.55–132.99, P value .001) and (OR 9, 95% CI; 1.50–53.86, P value .01) respectively.

The Risk association of Lung cancer in relation to Fas and FasL genotypes with smoking history and clinical characteristics of patients was demonstrated in Table 4 and showed that the non smoking patients having the FasL-CC genotype have 2.25 folds increased risk to develop lung cancer (OR 2.25; CI: 1.08–4.67, P value .008). No correlation was found between the pathological types of lung cancer nor the stages and the Fas and FasL genotypes.

Table 1
Distribution of patients and controls characteristics.

	Patients (n = 20)		Controls (n = 40)		P-value	***OR (95% CI)
	No	%	No	%		
Gender					.17	0.4 (0.1–1.5)
Male	14	70	34	85		
Female	6	30	6	15		
Smoking						
Non-Smokers	9	45	20	50		
Smokers	11	55	20	50	.72	1.2 (0.4–3.6)
Pack/year	**(n = 11)**		**(n = 20)**			
<20	0	0	11	55		
≥20	11	100	9	45	**<.05**	2.2 (1.4-3.6)
Histological type						
SCC*	8	40				
Adenocarcinoma	5	25				
Other**	7	35				
Stages						
II	4	20				
III	10	50				
IV	6	30				

* Squamous cell carcinoma (SCC).
** Other includes, 2 large cell, 2 spindle cell, 3 undifferentiated. P-value < .05 is statistically significant.
*** Odd's ratio.

5. Discussion

Single-nucleotide polymorphisms (SNPs) have been recognized in the promoter region of the Fas gene, A or G at position-670 (Fas-670A/G) and G or A at position-1377 (Fas-1377G/A). Reduced promotor activity and paucity of Fas gene expression represent a consequence of the disruption of TAT1 and Sp1 transcription factor-binding sites by Fas-670G allele and the Fas-21377A allele respectively [20,21]. Regarding functional SNPs of FasL promoter activity, T or C at position 2844 (FasL2844T/C) have been demonstrated, where the FasL2844 C allele is significantly linked with higher basal expression of FasL than the FasL2844 T allele [22]. The Fas21377G/A, and FasL2844T/C polymorphisms might be allied with the increased risk of lung cancer.

In the present study, the frequency of the homozygous mutant allele Fas-AA was higher among the patient group compared to control and showed 5.28 folds increased risk of lung cancer. Upon adjustment of this comparison as regards the sex, smoking history and the smoked packs per year, both Fas AA & GA genotypes demonstrated statistically significant differences between the two groups where the carriers of these genotypes have 10 and 41 folds increased risk of lung cancer, respectively. Meanwhile, FasL genotypes did not differ significantly between patient and control groups. In agreement with ours, Zhang et al. (2005) demonstrated that carriers of the Fas 21377AA genotype have increased risk of lung cancer by 1.6 folds [18]. Also, Park et al. (2006) and Sung et al. (2011) reported that the FasL 2844T/C genotype was not linked with increased lung cancer risk [17,23].

Gene-gene interaction was implicated in this study in order to highlight the role of FasL2844T/C gene polymorphism. The combination of the homozygous mutant alleles FasL CC with the homozygous Fas AA mutant alleles or either the wild GG allele and heterozygous GA were more frequent in patients (30% and 25% respectively) compared to that in control (20% and 5% respectively) and demonstrating 9 and 72 folds increased risk to develop lung cancer. Similarly, the combination of the homozygous Fas AA with the wild and heterozygous FasL TT, CT was more frequent in patients (40%) compared to that in control (12.5%) increasing the risk of lung cancer by 10.28 folds. These findings were in accordance with Zhang et al. (2005) where they assumed that patients with the FasL 2844CC genotype have more chance to carry Fas 21377AA than the controls were, but with only 4.18 folds increased risk to develop lung cancer [18]. Similarly, numerous studies announced that subjects carrying both Fas 21377AA and FasL 2844CC could be at higher threat for acquiring lung cancer than those carrying either the Fas 21377AA or FasL 2844CC alone [24–28]. Gene–gene interaction of Fas and FasL polymorphisms

Table 2
The distribution of Fas and FasL genotypes frequencies among patients and controls and their associations with the risk of lung cancer.

Genotype	Controls (n = 40)		Patients (n = 20)		OR (95% CI)	P value	OR (95% CI)	P value
	No.	%	No.	%	Crude	Crude	Adjusted	Adjusted
Fas								
AA	13	32.5	14	70	5.28 (1.35–20.65)	0.01	10 (2.0–50.23)	0.005
GA	1	2.5	2	10	9.48 (0.63–141.8)	0.1	41.48 (1.04–164.1)	0.04
GG	26	65	4	20	1 (reference)	–	1 (reference)	–
FasL								
CC	10	25	11	55	2.32 (0.61–8.74)	0.2	1.67 (0.37–7.42)	0.49
CT	3	7.5	2	10	1.83 (0.21–15.4)	0.5	2.38 (0.22–24.82)	0.46
TT	27	67.5	7	35	1 (reference)	–	1 (reference)	–

P-value < .05 is statistically significant.*Odd's ratio.

Table 3
The combined effect of Fas and FasL genotypes on the risk of the lung cancer among patients and controls.

Genotypes					
FasL 2844	Fas 21377	Patients, n (%)	Controls, n (%)	OR* (95% CI)	P-value
TT+CT	GG +GA	1 (5%)	25 (62.5%)	1 (reference)	–
TT+CT	AA	8 (40%)	5 (12.5%)	10.28(1.68–62.74)	.01
CC	GG +GA	5 (25%)	2 (5%)	72 (5.55–132.99)	.001
CC	AA	6 (30%)	8 (20%)	9 (1.50–53.86)	.01

P-value < .05 is statistically significant.
* Odd's ratio.

Table 4
Risk association of Lung cancer in relation to Fas and FasL genotypes with smoking history and clinical characteristics of patients.

	Fas 21377 genotype				FasL 2844 genotype			
	AA (n) patients/controls (14/13)	GG+GA (n) patients/controls (6/27)	OR_(95% CI)	p value	CC (n) patients/controls (5/34)	TT+TC (n) patients/controls (15/6)	OR_(95% CI)	p value
Smoking status								
Nonsmoker	(7/8)	(2/12)	0.5 (0.068–3.67)	.64	(4/3)	(5/17)	2.25 (1.08–4.67)	.008
Smoker	(7/5)	(4/15)	0.5 (0.13–1.93)	.5	(11/3)	(0/17)	1 (0.17–5.67)	1
Pack years smoked								
<20	(0/7)	(0/4)			(0/5)	(0/6)		
≥ 20	(7/3)	(4/6)	0.44(0.05–3.5)	.61	(6/2)	(5/7)	0.35 (0.02–4.65)	.56
Histological type								
SCC*	(6/–)	(2/–)		.1	(5/–)	(3/–)		.2
Adenocarcinoma	(5/–)	(0/–)			(1/–)	(4/–)		
Other_	(3/–)	(4/–)			(5/–)	(2/–)		
Stages								
II	(1/–)	(3/–)		.06	(2/–)	(2/–)		.4
III	(9/–)	(1/–)			(8/–)	(2/–)		
IV	(4/–)	(2/–)			(5/–)	(1/–)		

* Squamous cell carcinoma (SCC). P-value < .05 is statistically significant. *Odd's ratio.

augment the risk of lung cancer development in a multiplicative mode. The altered cells with high level of FasL as a result of the FasL 2844CC genotype expression may evade host immune surveillance either by creating an immune privileged site and/or killing cytotoxic immune cells [18].

Tobacco smoking is a conventional, widely known causal factor for lung cancer. In this study it was found to be prevalent by 1.2 times more among smokers than among the non-smokers and to be 2.2 times more risky in heavy smokers (≥20 pack/year) than in light smokers (<20 pack/year). Gene–environment interaction of Fas or FasL polymorphisms and smoking coupled with increased risk of lung cancer was found in several studies. Although our study did not demonstrate significant difference regarding FAS gene polymorphisms and smoking, yet Zang et al. (2005) announced that the risk of lung cancer was altered among smokers carrying Fas 21377AA genotype, while among the non smokers with the same genotype was not; signifying a gene–environment interaction. However, this involvement was higher in light than in heavy smokers where tobacco carcinogens are giving rise to transformed or pre-invasive lung cells and when coupled with either low Fas expression and/or increased FasL, one of these cells will evade immune surveillance to become carcinogenic [18]. For the FasL polymorphism, in this study the increased risk to develop lung cancer by 2.25 folds was demonstrated among non-smokers carrying the FasL-CC genotype. Also, Zhao et al. (2016) observed that the risk of lung cancer was increased significantly among both smokers and non-smokers with more pronounced increase among heavy smokers than light smokers [29]. Moreover, because tobacco smoking can provoke FasL expression Suzuki et al. (1999) and Bijl et al. (2001) stated another hypothesis for this relation, where the level of FasL expression from the FasL 2844C allele was higher than that from the FasL 2844T allele upon induction by smoking and in addition to the higher constitutive expression resulting from the FasL 2844T/C polymorphism, smoking and FasL 2844CC genotype increase vulnerability of lung cancer [30,31]. Zhang et al. (2005) stated that the high level of environmental smoke that non-smokers exposed to throughout ordinary life may justify the higher risk of lung cancer among non smokers carrying FasL 2844CC genotype when compared with those carrying the same genotype and smoked less than 20 packs per year [18].

The results comparing lung cancer vulnerability with the Fas and FasL polymorphisms among different subtypes and stages of lung cancer were controversial. In this study, neither the histological type of lung cancer nor its stage have an impact on the risk of developing lung cancer when correlated with 21377AA genotype or FasL 2844CC genotype. Similar findings were declared by Zhang et al. (2005) when correlating the risk of lung cancer with Fas 21377AA or FasL 2844CC genotypes and histological types of lung cancer [18]. Conversely, Zhao et al. (2015) & (2016) found that, FasL 2844 and Fas 21377 SNPs were linked with increased vulnerability of pulmonary adenocarcinoma (AD) [5] and FasL 2844CC was found to be a risk factor for pulmonary SCC [29]. Though Sung et al. (2011) did not find that FasL 2844 polymorphism increased the risk of lung cancer, they found that FasL 2844CC genotype had higher incidence in those with advanced tumors than in those with early tumors, also FasL 2844CC was found to be a risk factor

for lymph node metastasis and vascular tumour thrombus [23]. This may support the possibility that pulmonary SCC with FasL 2844CC has a stronger invasion [29]. Findings related to the genes-smoking interaction, the histopathological types and the stage of lung cancer were not always consistent with one another, partially because of dissimilarity in sample sizes and cultural backgrounds.

In conclusion, this study provides the substantiation that functional Fas and FasL polymorphisms are associated with the increased risk of lung cancer and exhibit a gene–gene interaction. The FasL polymorphism increases the risk of lung cancer in non-smokers.

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