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Review

Fas and Fas Ligand polymorphisms in human cancer: their effect in cervical cancer

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ABSTRACT

Apoptosis is the major component of programmed cell death, an essential process in embryogenesis, tissue turnover and proper function of the immune system. Lack of appropriate control is thought to play an important role in several pathologies, such as autoimmune diseases, AIDS and cancer. In malignant tumors, cells gradually acquire resistance to apoptosis and, moreover, develop mechanisms that could induce death cell in immune cells. Emerging interest has given to the Fas/Fas Ligand interaction, a system that triggers the extrinsic pathway of the apoptosis process. Polymorphisms on Fas and FasL have been extensively described. Single nucleotide polymorphisms located in the promoter region of these genes have been found to be related to differential levels of expression. This review discusses the information published on Fas/Fas Ligand polymorphisms and its effect on human cancers, and also presents new data regarding the impact of Fas-670A/G and FasL-844T/C polymorphisms in a cervical cancer case-control study from women of La Plata, Argentina.

KEYWORDS: Fas/Apo-1, Fas Ligand, cancer susceptibility, cervical cancer

INTRODUCTION

During body development and tissue turnover cells proliferate, differentiate and die in a highly controlled, regulated fashion. There are at least three types of cell death: autophagy, necrosis and apoptosis. In contrast to necrosis, in which uncontrolled cell death leads to lysis and inflammatory responses, apoptosis is crucial for normal growth and it is the inherent process in the thymus that eliminates self reactive pre T cells. It can be initiated by a diverse range of pro-apoptotic signals. They can be originated intracellulary, in response to cell stress, or extra cellular, if death inducing signals bind to cell surface receptors, like Fas(TNFR6), TNFR1 and TNFR2 [1].

Early indications of Fas activity date back to the late 80's, when Yonehara *et al*. (1989) described a cell-killing monoclonal antibody with cytolytic activity, called anti-Fas, whose action was indistinguishable from TNF in several human cell lines [2]. Almost simultaneously, Trauth *et al*. (1989) described a mouse antibody called anti-Apo-1 that targeted a cell surface protein on activated lymphocytes and human lymphoma cell lines and prevented growth by induction of apoptosis [3]. Identification of the surface antigen was accomplished by Itoh *et al*. (1991), who isolated Fas cDNA from T cell lymphoma KT-3 cells, and found that the predicted molecule was a transmembrane protein of 319 amino acids and 36 kD in weight [4]. A year later, Oehm *et al*. (1992) purified the target of anti-Apo-1 antibody from SKW6.4 cells, and discovered that the resultant protein, APO-1, was the same as Fas [5].

Fas gene is located on chromosome 10q24.1, encompassing nine exons and eight introns. It encodes for a Type 1 transmembrane protein with

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three cisteine-rich extracellular domains (exons 1-3), a transmembrane domain (exon 6), and three intracellular domains (exons 7-9) [6]. Receptors with the ability to trigger the extrinsic pathway of apoptosis are collectively called "death receptors", and involve members of the TNF Receptor super family (TNFR6/Fas/Apo-1, TNFR1, TNFR2, DR3, DR4, DR5) [7]. Death receptors share a homologous cytoplasmic motif of about 80 amino acids in length, which is responsible for transducing death signals, called the death domain (DD) [6]. Expression analyses revealed that Fas is expressed on the cell surface of fibroblasts, myeloid cells and T lymphoblastoid cells. Fas mRNA is also detected in mouse thymus, ovary, heart and liver, but not in brain and spleen [8].

Some time later the ligand that triggers apoptosis was identified by anchorage to the Fas receptor on the surface of a cytotoxic T cell hybridoma. The obtained cDNA was identified by expression cloning and the product indicated a type II transmembrane protein of 280 amino acids that belongs to the TNF family [9]. Further analyses revealed that Fas Ligand (FasL) maps on chromosome 1q23, consists of approximately 8 kilobases and it is divided in 4 exons. Consistent with its involvement in T cell-mediated cytotoxicity and in several non-lymphoid tissues, FasL is expressed in activated splenocytes and thymocytes [9, 10].

The binding of FasL to its receptor occurs via cell-cell contact and induces the trimerization of Fas receptor in the cellular membrane. Fas/FasL interaction promotes the formation of a cytoplasmic complex, called death inducing signaling complex, or DISC [11]. Upon Fas trimerization, death domains tend to aggregate, and allow recruitment of a cytoplasmic adaptor protein, the Fas associated death domain (FADD) protein. FADD also has a death effector domain (DED) that interacts with the death domain of Fas, promoting pro-caspase 8 recruitment and its activation by self-cleavage [12, 13]. A protease cascade is triggered by various members of the caspase family. Eventually, DNA degradation and enzymatic digestion of several cell targets will lead to cell death [14].

Fas and Fas Ligand changes have been suspected to be associated with autoimmune diseases and cancer. Mutations in these genes have been demonstrated to be the cause of autoimmune lymphoproliferative syndrome (ALPS), a very rare condition characterized by lymphoadenopathy, hepatosplenomegaly and autoimmune disease [15]. The contribution of "normal" allelic variants (polymorphisms), however, is an emerging area of research. It is the aim of this review to outline aspects of the current literature focused in the Fas/FasL proteins, with special attention to cervical cancer.

Genetic variability

To date, approximately 70 pathological changes have been reported for the Fas gene. Most of them correspond to deletions or non sense mutations that cause the above mentioned ALPS condition [15]. Until now, more than 290 single nucleotide polymorphisms were reported across the Fas Open Reading Frame. However, only thirteen SNPs belong to exonic DNA, with seven of them encoding missense changes [16]. Researchers have given special attention to variations located in the 5' regulator sequences. In this sense, two single nucleotide polymorphisms have been widely studied. A G to A transition at position -1377 (Fas-1377G/A, [rs2234767]), which is located within the consensus sequences of the *stimulatory protein 1* (SP-1), and an A to G change located at position -670 (Fas-670A/G, [rs1800682]), which resides in the *signal transducer and activator of the transcription 1* (STAT1) transcription factor binding site. It is believed that these two polymorphisms may dis-regulate Fas promoter activity and affect proper gene expression by hampering binding of their respective transcription factors [17, 18, 19]. Since FasL has a smaller sequence, only 55 SNPs have been described so far, most of which are located in introns and remain to be validated by further studies. Eight SNPs belong to exonic DNA, with six of them encoding missense changes [16]. Similar to Fas, the promoter is being widely studied, in special the -844 T to C substitution (FasL-844T/C, [rs763110]). This SNP is located within a putative binding motif which is recognized by a transcription factor, the *CAAT/enhancer binding protein beta* [20].

Fas/FasL and cancer risk

Two recent meta-analyses focused on the effect of Fas promoter polymorphisms in overall cancer risk have been recently published. Qiu *et al*. (2009) pooled data from 17 studies, including 10,564 cases and 12,075 controls. There was a slight, but significant, elevated risk for the Fas-1377AA genotype (AA vs AG, OR=1.19; AA vs AG+GG, OR=1.21) [21]. When the analysis was stratified, Asian ethnicity and breast cancer showed a borderline but statistically significant association for the homozygous AA genotype (AA vs GG, OR=1.29). Similar results were found by Zhang *et al*. (2009), who examined Fas-670A/G and Fas-1377G/A polymorphisms in 34 case-control studies. A slightly increased risk was found among patients who carried the Fas-1377AA genotype (AA vs GG, OR=1.2; AA vs GA+GG, OR=1.23), specially in smokers (AA vs GA+GG, OR=1.96). Non significant higher risk was found for Fas-670A/G polymorphism. In agreement with Qiu, Asian ethnicity and breast cancer showed a significant higher cancer risk among A allele carriers, but carriers with melanoma had a significant decreased risk [22].

The interaction between FasL-844C/T and cancer susceptibility has been explored recently. Liu *et al.* (2009) pooled data from 18 association studies and found a slightly increased risk for FasL-844CC carriers (CC vs TT, OR=1.23; CC vs CT+TT, OR=1.20). Interestingly, higher risks were reported for Asians (CC vs TT, OR=1.6), and population based case-control studies [23]. Another meta-analysis published the same year gathered information from 19 association studies and comprised 11,105 cases and 11,372 controls. The results were similar to the previous study. FasL-844TC and TT genotypes conferred significantly lower risks for cancer than the CC genotype (CT+TT vs CC, OR=0.82), the highest effect was obtained in smoking-related cancers and Asian ethnicity [24].

Besides the meticulous associations reported by the above-mentioned meta-analyses, common obstacles reported by the authors were: the number of studies, which is still low for any given cancer type; and studies heterogeneity, a problem that can arise when attempting to undertake global data. However, recent years have witnessed that the number of reports is increasing, and almost every cancer site has been studied for a relationship with Fas pathway polymorphisms. The following malignancies were selected because they present either new or interesting data about a potential role of Fas SNPs in cancer susceptibility.

In vivo and *ex vivo* T cell assays support the view that functional Fas/FasL polymorphisms may act as low penetrance genes in breast cancer, particularly if external factors are considered. A well-characterized case-control study performed in China (840 cases and 840 controls) revealed that patients harboring the Fas-1377AA or GA genotypes present moderately increased risk compared to those harboring the GG genotype. Also, FasL-844CT and FasL-844TT had a significantly lower risk compared to those carrying the CC genotype [25]. In contrast, Crew *et al*. (2007) did not find significant differences among Fas-670A/G, Fas-1377G/A and FasL-844T/C genotypes and breast cancer cases, although it is worth mentioning that Fas SNPs were associated with breast cancer in women with detectable polycyclic aromatic hydrocarbon (PAH)-DNA adducts [26].

In addition, there is evidence of a potential link between Fas polymorphisms and subtypes of acute myeloid leukemia (AML). It is believed that deregulation of the Fas pathway confers an increased risk for hematological malignancies. A well-controlled study developed in the United Kingdom reported elevated risk for developing AML in carriers of the Fas-1377A allele, Fas-1377GA or AA genotypes (OR=1.69), or Fas-1377A/Fas-670A haplotypes [19]. However, Fas genotypes were not associated with AML in South Korea patients [27], and it does not seem to influence disease outcome in children with AML [28].

Melanoma studies have provided controversial findings over Fas SNPs. While one study from the USA reported that individuals carrying Fas-1377GG (OR=1.32), and Fas-670AA (OR=1.28) genotypes are at an increased risk for melanoma cancer [29], researchers from Sweden did not detect an association between Fas/FasL polymorphisms and cancer risk. It should be mentioned, however, that such polymorphisms

could be potential markers for the development and progression of sun-induced melanoma [30].

Colorectal cancer has been recently revised in a longitudinal study from Austria, where 433 patients were retrospective evaluated. Carriers of Fas-670GG genotype had significantly lower survival rate than those with AG/AA genotypes (RR=1.76) [31]. Further evidence on Fas-670A/G polymorphism has been reported for nasopharyngeal cancer (NPC) patients in Tunisia. Patients carrying Fas-670AG (AG vs AA, OR=2.00) and Fas-670GG (GG vs AA, OR=3.19) genotypes were shown to be at higher risk for NPC. Moreover, Fas polymorphism was associated with induction of nuclear auto-antibodies, suggesting a role in immune deregulation of cancer [31]. Although another case control study from China could not confirm the association with NPC, lymph node dissemination and metastasis were increased among carriers of Fas-670AG+GG genotypes and the Fas-670G allele [32].

There is experimental evidence that Fas polymorphisms are associated to tobacco exposure and therefore may have impact on lung cancer risk. Wang *et al*. (2003) demonstrated that Fas alleles differentially modulate the apoptotic capacity of cultured peripheral blood lymphocytes in response to exposure to tobacco carcinogen [33]. In this sense, Zhang *et al*. (2005) showed that individuals carrying the Fas-1377AA (OR=1.59) and FasL 844CC (OR=1.79) genotypes are at higher risk of lung cancer [34]. However, other authors failed to find an association between Fas/FasL polymorphisms and lung cancer [35, 36, 37].

The relationship between Fas/FasL polymorphisms and non-small cell lung cancer (NSLC), a type of lung cancer which is commonly not associated with smoking, has been recently revised in a large case control study (2,644 cases and 1,619 controls) from USA. Although there was non-significant association for Fas/FasL polymorphisms, a moderate risk (OR=1.58) was found in subjects under 60 with the FasL-844T/C heterozygous genotype compared to the CC genotype [38]. On the other hand, a longitudinal study conducted in South Korea over 338 patients with NSLC found that Fas-670GG genotype and G allele may be used as useful prognostic markers for survival in early disease [39].

Genes Fas/Fas Ligand in cervical cancer

Cervical cancer is caused by long term Human Papillomavirus (HPV) infection. However, only a few numbers of women with HPV infection will develop cancer, suggesting that additional factors should be involved. Among genetic candidates, experimental findings indicated that death pathway genes Fas and FasL may be associated to cancer susceptibility. Tumor specific cytotoxic lymphocytes often disappear before a cervical tumor is eliminated via Fas induced apoptosis. Indeed, cervical tumor tissues and cervical cancer lines commonly express Fas receptor, inducing death of cytotoxic T cells [40, 41].

The relationship between Fas/FasL polymorphisms and cervical cancer varies among populations and laboratories. Highest risks were seen in populations from Asiatic countries. In this sense, Sun *et al*. (2005), who examined 314 cases and 628 controls from China, found that patients carrying the FasL-844CC genotype are at higher risk of cervical cancer than patients carrying the FasL-844TT genotype. These observations correlated with experimental findings. A considerably higher expression of FasL was found among lymphocytes with the FasL-844CC allele compared with the CT or TT allele, but not for Fas polymorphisms. Moreover, FasL-844CC was associated with enhanced rate of apoptosis induced cell death in T cells, suggesting that this polymorphism may be acting as a contributing factor for cervical carcinogenesis [42].

Lai *et al*. (2003) reported a significant risk for cervical cancer in another case control study from China (104 patients with low grade SIL, 131 high grade SIL, 176 SCC and age-matched controls). They found that the frequency of Fas-670AA genotype and the A allele increased in accordance with the multistep model from cervical intraepithelial lesions to invasive squamous cell cancer. Individuals carrying the AA genotype had a higher risk for developing HSIL (OR=1.3) and SCC (OR=1.6) than the GG genotype [43]. Paradoxically, a recent study conducted on a cohort of 354 Japanese women with diverse gynecological cancers found that the Fas-670GG genotype and the G allele were statistically higher in cervical cases than in controls (GG vs AA, OR=2.51; G vs A, OR=1.6) [44]. Contrary to

these results, there was not a significant difference among 150 cervical cancer cases and 160 healthy controls from a population from South Korea, although Fas-1377GA or AA genotypes showed an increased incidence in patients with nodal metastasis [45]. In India, another positive association between cervical cancer and Fas-670 polymorphism was found. The heterozygous AG genotype showed a highly significant risk for cervical cancer when compared to the AA genotype (OR=3). Indeed, the combined AG+GG genotypes showed significant higher risk for cervical cancer development, suggesting a dominant model of action (OR=2.54). The estimated risk was even higher when the analysis was restricted to passive smokers [46].

On the other hand, studies from Europe did not find association between Fas polymorphisms and cervical cancer. In this sense, Engelmark and coworkers (2004) could not find a relationship between the Fas-670A allele and *in situ*, cervical cancer in a study performed in Sweden. They analyzed 278 affected sib-pairs (ASPs) with cervical cancer using maximun lod-scores (MLS) values, and found that ASP did not differ significantly from random, even when the analysis was stratified on the basis of human leukocyte antigen (HLA) class II susceptibility DQB1*0602/ DRB1*1501 haplotypes [47]. Similar results were obtained in Poland, where Fas-670A/G polymorphism distribution was similar in cases and controls [48].

Studies exploring Fas/FasL polymorphisms in South American populations are scarce. A recent case control study from Brazil reported a non-significant difference between patients with cervical cancer and the Fas-670A/G polymorphism. However, the heterozygous genotype was increased in younger patients (less than 48 years old; OR =0.85); when compared with the wild type [49].

A case control study in a population from Argentina

In order to elucidate the potential role of Fas-670A/G and FasL-844T/C polymorphisms in cervical cancer, a case-control study was performed in 193 controls and 103 SCC patients from the city of La Plata, Argentina. HPV detection was assessed by a nested PCR approach, using My and GP+ primers [50, 51], and genotyped by HPV 16 and 18 type-specific primers (E616F 5'-gag aac tgc aat gtt tca gga cc-3'; E616R 5'-cct cac gtc gca gta act gtt gc-3'; E618F 5'-aga gac agt ata ccc cat gct-'3; E618R 5'-gtt tct ggc acc gca ggc acc t-3'). The PCR mix was constituted of: 1.5 uL of each primer (12.5 pmol/uL); 4 uL of 0.5 mM dNTPs; 0.15 units of Taq polymerase (Invitrogen, USA); 2.5 uL commercial buffer; 3 mM of Cl_2Mg; 5 uL of sample DNA; and distilled water up to 25 uL. Cycling conditions were: 4' at 94º; 35 cycles of 1' at 92ºC, 1' at 58ºC, 1' at 72ºC; and 5' at 72ºC. The amplified products yielded 134 bp and 164 bp fragments for HPV 16 and 18, respectively.

Fas-670A/G polymorphism was determined by traditional PCR-RFLP. Gene amplification was performed by the following oligonucleotides: FasF 5'-cta cct aag agc tat cta ccg ttc-3'; FasR 5'-ggc tgt cca tgt tgt ggc tgc-3'. The PCR product was 332 bp long. The PCR mix was constituted of: 2 uL of each primer (12.5 pmol/uL); 3 ul of 0.5 mM dNTPs; 0.15 units of Taq polymerase (Invitrogene, USA); 5 uL of commercial buffer; 8 mM of Cl_2Mg; 3 uL of 100mg BSA; 5 uL of sample DNA; and distilled water up to 50 uL. Cycling conditions were: 4' at 94º; 35 cycles of 30'' at 92ºC, 40'' at 62ºC, 40'' at 72ºC; and 5' at 72ºC. After cycling, the amplicon was digested overnight at 37°C with MvaI. Enzyme digestion yielded bands of 100 and 232 bp for the A allele and 332 bp for the G allele.

FasL polymorphism was determined by pyrosequencing technology, as described in [52]. The primers used for were: FasLF 5'-ctg cta cac cca ctt tag aaa tta ga-3'; FasLR 5'-ggg caa aca atg aaa atg aaa aca tcg -3'; and for sequencing FasIn 5'-aga gct gct ttg tatt-3'. The PCR mix was constituted of: 2.4 uL of each primer (12.5 pmol/uL); 4 uL of 0.5 mM dNTPs; 0.2 units of Taq polymerase (Invitrogene, USA); 5 uL of comercial buffer; 3 mM of Cl_2Mg; 3uL of 100mg BSA; 5 ul of sample DNA; and distilled water up to 50 uL. Cycling conditions were: 4' at 94º; 35 cycles of 30'' at 92ºC, 40'' at 57ºC, 40'' at 72ºC; and 5' at 72ºC. PCR amplification yielded a 100 bp long amplicon, which was subsequently sequenced in a 96MA pyrosequencer (Biotage TM).

Table 1. Crude and adjusted risk estimates for Fas-670A/G and FasL-844T/C polymorphisms for cervical cancer from women of La Plata, Argentina.

Fas -670A/G	Number		OR (IC95%) p value	OR age (IC95%) p value	OR age HPV* (IC95%) p value
	controls	cases			
GG	46	18	Ref.	Ref.	Ref.
GA	101	56	1.417 (0.751-2.674) NS	1.035 (0.507-2.115) NS	1.182 (0.511-2.731) NS
AA	46	29	1.611 (0.787-3.297) NS	1.658 (0.753-3.650) NS	1.145 (0.453-2.894) NS
FasL -844T/C					
CC	67	42	Ref.	Ref.	Ref.
CT	96	50	0.831 (0.496-1.391) NS	0.777 (0.429-1.406) NS	0.638 (0.309-1.318) NS
TT	30	11	0.585 (0.265-1.290) NS	0.552 (0.222-1.373) NS	0.367 (0.129-1.038) NS

NS non significant p value.
*HPV adjusted ORs by HPV 16 and 18 positivity.

The association of variables was performed by chi square test. Adjustment of confounders and risk estimations were determined by logistic regression. Gene interactions and/or epistasis were estimated by Multifactorial Dimensionality Reduction (MDR) and ED (Enthropy Decomposition) [53].

A summary of the results is shown in Table 1. The corresponding allele frequencies for the Fas-670A allele was 0.5 in controls and 0.55 in cases, while the FasL-844C allele was 0.6 in controls and 0.65 in cases. The control population reached Hardy-Weinberg equilibrium for both markers. As seen in the table, there has not been a statistically significant excess in risk for cervical cancer considering Fas-670A/G or FasL-844T/C SNPs genotypes, in raw or adjusted calculations.

Further analyses were performed in order to evaluate a potential gene-gene interaction. MDR is a non-parametric approach specifically designed to evaluate gene-gene interactions, model free and based on inductive construction algorithms [53]. According to the obtained data, the combination of genotypes from Fas-670A/G and FasL-844T/C polymorphisms did not statistically differ from random (Balanced Accuracy, 0.55; Sensitivity, 0.46; Specificity, 0.65; Odds Ratio, 1.6339 (0.9623, 2.7742); Precision, 0.39). Entropy decomposition analysis showed a near null interaction between both SNPs and cervical cancer risk.

CONCLUSIONS

Mutations in Fas and FasL can contribute to cell transformation by hampering the apoptotic signal transduction pathway, leading to loss of balance between proliferation and cell death. There is strong evidence that indicates that defective Fas is the cause of autoimmune lymphoproliferative syndrome and certain lymphomas [15]. On the other hand, the contribution of genetic polymorphisms to the individual susceptibility has drawn increasing attention to complex malignancies, mainly because it could explain a component of population variability. In this context, Fas and FasL SNPs emerge as promising candidates.

According to the literature, polymorphisms in the Fas gene may have a weak effect in autoimmune diseases, in particular Multiple Sclerosis and Systemic Sclerosis [54, 55]. The association is biologically plausible, since Fas chromosomal location is linked to multiple sclerosis transmission in affected families, and alterations in Fas lead to a lupus erythomatosus-like disease

in mice [56, 57]. On the other hand, a growing number of studies have suggested that Fas/FasL polymorphisms may act in genetic susceptibility to cancer. Among the functional markers, Fas-670A/G polymorphism seems to be the most controversial. Some authors have found slightly higher risks for skin cancer, lung cancer, and cervical cancer, while others have not. In the present case control study, Fas-670 was not statistically associated to cervical cancer, and adjusted odds ratios were close to the unity. Besides the fact that the number of studies for each cancer is low, the current evidence is in consistence with experimental findings. Electrophoretic mobility assays demonstrated that Fas-670A/G alleles does not differ in ability to bind to the transcription factor STAT1 [18, 19], and therefore it is expectable to deliver a comparable impact on cancers. However, a potential role for Fas-670A/G polymorphism should not be dismissed in further studies, since *in vivo* interactions are more complex than experimental assays. In addition, Fas-670A/G polymorphism was suggested as a predicting marker for survival in NSLG if adjusted for clinic pathological factors [19], and might promote Fas aberrant expression by interaction between STAT1 and SP1 transcription factors (Fas-1377G/A) [58].

Several lines provided evidence that supports FasL-844T/C and Fas-1377G/A alleles as low penetrating genes for certain types of cancer. Fas-1377G/A polymorphism seems to be a contributor to genetic susceptibility in breast cancer, while FasL-844CC might contribute to genetic susceptibility to overall cancer, specially in smoking-related malignancies and Asian ethnicity. Similar to these data, the present study found that FasL-844TT and CT genotypes had a lower risk for cervical cancer than FasL-844CC, although the numbers did not reach statistical significance. Moreover, when important confounders for cervical cancer, such as age and high risk HPV infection are taken into account, the odds ratio for FasL-844TC and TT is even lower. The obtained results reflect previous biological findings. It has been shown that in normal cervical tissues Fas Ligand expression is confined to the basal layer, but in tumors the expression pattern changes completely: FasL is expressed in most of the carcinoma cells, and more than 90% of the observed apoptosis belong to leukocytes [59]. The aberrant production appears to be a malignant attempt to destroy host's lymphocytic reaction [41].

To better understand the contribution of Fas/FasL polymorphisms in complex diseases, more and larger studies are necessary, including stratified analyses. It is necessary to include confounders as well as potential gene-gene interactions, with sufficient statistical power to discriminate linkage disequilibrium due to association and not linkage.

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