

## ***IFNG* Polymorphism (+874 T>A) is not a risk factor for cervical cancer**

**Ani Melani Maskoen\*\*\*, Herman Susanto\*\*\*, Samsudin Surialaga\*, and Edhyana Sahiratmadja\*\*\***

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### ABSTRACT

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#### **INTRODUCTION**

Cervical cancer cases are rising and many women are infected with human papillomavirus (HPV). Interferon gamma (IFN- $\gamma$ ) is one of the key regulatory cytokines that influence the HPV clearance. The production and the function of IFN- $\gamma$  may be impaired by the defect of the IFNG gene leading to the cervical malignant progression. This study aimed to examine the association between IFNG+874 T>A polymorphism and cervical cancer in women

#### **METHODS**

In a case-control study design, consecutive untreated women with cervical cancer who showed for the first time in Hasan Sadikin Hospital Bandung were enrolled (n=98) and for controls women who came for PAP smear (n = 81). Controls were not matched in ages and ethnicities. DNA extracted from blood was amplified by amplification refractory mutation system - polymerase chain reaction method (ARMS – PCR) to detect IFNG+874 T>A polymorphism.

#### **RESULTS**

The distribution of IFNG genotypes TT, TA and AA for women with cervical cancer who met the inclusion criteria (n= 64) and with negative intraepithelial lesion or malignancy (n=42) were 14.1%, 50.0%, 35.9% and 7.1%, 52.4%, 40.5%, respectively. No significant differences could be observed between both groups (p=0.64). Stratifying the cervical cancer women into a group of squamous cell carcinoma (n = 54) revealed no statistical difference.

#### **CONCLUSION**

IFNG +874 T>A polymorphism seems not to contribute in susceptibility to cervical cancer. Identification of other variants in IFNG gene signaling and its role in the development of cervical cancer diseases need to be further examined.

**Key words:** IFNG gene, single nucleotide polymorphism, cervical cancer

\*Department of Biochemistry,  
Faculty of Medicine,  
Universitas Padjadjaran,  
Bandung

\*\*Health Research Unit,  
Faculty of Medicine,  
Universitas Padjadjaran,  
Bandung

\*\*\*Department of Obstetrics  
and Gynaecology,  
Hasan Sadikin Hospital/  
Faculty of Medicine,  
Universitas Padjadjaran,  
Bandung

#### **Correspondence**

Dr. Ani Melani Maskoen, drg,  
MKes.

Department of Biochemistry /  
Health Research Unit  
Faculty of Medicine,  
Universitas Padjadjaran,  
Bandung, Indonesia  
Jl. Eijkman No. 38 Bandung  
Phone: + 62 22 2038218  
Email : amelani@yahoo.com

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## ***Polimorfisme IFNG+874 T>A bukan merupakan faktor risiko untuk kanker serviks***

### **ABSTRAK**

#### **PENDAHULUAN**

Walaupun banyak wanita yang terinfeksi virus human papilloma (HPV) tetapi hanya sebagian saja dari wanita yang secara kronik terinfeksi oleh HPV tipe risiko tinggi akan berkembang menjadi kanker serviks. Interferon gamma (IFN- $\gamma$ ) merupakan salah satu sitokin yang berperan dalam mengeliminasi HPV. Produksi dan fungsi IFN- $\gamma$  dapat mengalami penurunan bila terjadi perubahan pada gen IFNG yang menjadi sitokin IFN- $\gamma$  sehingga proses eliminasi HPV terganggu yang dapat menyebabkan kanker serviks dikemudian hari. Penelitian ini bertujuan untuk menentukan adanya asosiasi antara polimorfisme IFNG+874 T>A dan kanker serviks pada wanita.

#### **METODE**

Penelitian ini merupakan penelitian kasus-kontrol yang mana kasus adalah wanita yang datang ke poli kebidanan Rumah Sakit Hasan Sadikin untuk pertama kali dengan kasus kanker serviks baru. Kontrol diambil dari wanita yang datang untuk pemeriksaan PAP smear. Kontrol tidak dipasangkan dengan usia dan etnisnya. Darah EDTA diambil untuk diekstraksi DNA nya, kemudian diamplifikasi dengan menggunakan metoda amplification refractory mutation system - polymerase chain reaction (ARMS – PCR) untuk mendeteksi polimorfisme pada IFNG+874 T>A.

#### **HASIL**

Distribusi genotype IFNG TT, TA dan AA pada wanita dengan kanker serviks adalah 14,1%, 50,0%, 35,9%, dibandingkan dengan kontrol dengan PAP smear negatif sebesar 7,1%, 52,4%, 40,5%. Secara statistik tidak didapatkan perbedaan yang bermakna ( $p=0,64$ ). Dengan melakukan stratifikasi untuk kanker serviks hanya kelompok squamous cell carcinoma juga tidak didapatkan perbedaan yang bermakna.

#### **KESIMPULAN**

Pada penelitian ini, polimorfisme IFNG +874 T>A tampaknya bukan merupakan faktor risiko yang berkontribusi pada kerentanan terhadap kanker serviks. Identifikasi variasi-variasi yang lain pada gen IFNG perlu di pelajari dalam hubungannya dengan perkembangan kanker serviks.

**Kata kunci:** Gen IFNG, polimorfisme, kanker serviks

## **INTRODUCTION**

Cervical cancer is the second most prevalent female cancer after breast cancer, affecting annually more than 150.000 women worldwide.<sup>(1)</sup> This cancer has become a significant health burden in low- and medium-resourced countries in sub-Saharan Africa, Latin America, South and South East Asia, as 80% of cervical cancers occur in developing countries.<sup>(1)</sup> In Indonesia, cervical cancer ranks first among

gynecological cancers.<sup>(2)</sup> Persistent infection with high-risk types of human papillomavirus (HPV) is strongly associated with development of cervical cancer.<sup>(3)</sup> In immunocompetent individuals HPV infection is a self-limited disease that can be cleared by the immune system, as HPVs have a unique mechanism in limiting the infection to the basal cells of stratified epithelium, the only place where they replicate.<sup>(4)</sup> Meta-analyses on HPV genotypes distribution have shown that many women with normal cytology

can also be infected with high-risk types of HPV in single or multiple infections.<sup>(5)</sup> Interestingly only few women will ever develop cervical cancer. A recent study using a mouse cervicovaginal challenge model has proven that the virus cannot initially bind to keratinocytes *in vivo*.<sup>(6)</sup>

Immunological components in cellular mediated immunity, in particular interferon gamma (IFN- $\gamma$ ), play a key role in the development of cancers<sup>(4)</sup> and intracellular infections e.g. *M. tuberculosis*, *M. leprae*, and *Salmonella*.<sup>(7)</sup> IFN- $\gamma$  is one of the key regulatory cytokines, produced by activated T cells and natural killer (NK) cells, that enhances cellular immune responses by increasing T-cell cytotoxicity and NK-cell activity. IFN- $\gamma$  plays a role in antiproliferative, antitumor and antiviral activities. Furthermore, IFN- $\gamma$  is a pro-inflammatory cytokine playing a role in both innate and adaptive immune responses that may influence HPV clearance.<sup>(4)</sup> The susceptibility to HPV infection that leads to cervical cancer may be influenced by the gene that encodes IFN- $\gamma$ . Studies on HPV-infected patients point out the role of IFN- $\gamma$  in the control of the infection, for example low production or impaired function of IFN- $\gamma$  may lead to malignant progression of cervical HPV lesions.<sup>(8)</sup> Moreover, there is a direct relationship between IFN- $\gamma$  and the severity of cervical intraepithelial neoplasia.<sup>(8)</sup> Clearly, IFN- $\gamma$  plays a pivotal role in defense against viruses and intracellular pathogens and in the induction of immune-mediated inflammatory responses. Furthermore, intralesional IFN- $\gamma$  concentrations are detectable during HPV infections and may therefore be used as a valuable prognostic marker for clearance of high-risk HPV.<sup>(9)</sup> In the abovementioned study, after a 12-month follow-up period women who tested positive for the presence of IFN- $\gamma$  were significantly more likely to be free of HPV infection compared with women who were negative for IFN- $\gamma$ .<sup>(9)</sup>

The IFN- $\gamma$  gene (IFNG) encodes the cytokine IFN- $\gamma$ , thus host genetic differences in

the immune response to HPV infection may play a role in the susceptibility to disease.<sup>(10)</sup> A single nucleotide polymorphism located in the first intron of the *IFNG* gene can influence the secretion of the cytokine.<sup>(11)</sup> The *IFNG* genotype TT determines high *IFNG* production, and the *IFNG* genotypes TA and AA are for intermediate and low production of IFN- $\gamma$ , respectively.<sup>(11)</sup> Interestingly, ethnic background may influence the distribution of cytokine gene polymorphisms.<sup>(12)</sup> For example, studies in various populations have shown a clear correlation between ethnicity and distribution of *IFNG* polymorphisms across different population groups.<sup>(13)</sup> *IFNG*+874T>A polymorphisms are frequently studied in intracellular infectious disease such as tuberculosis<sup>(14)</sup> and also in cancers.<sup>(15)</sup>

Although meta-analyses of the *IFNG*+874T>A polymorphism have found it not to be associated with cancer in general, it still was associated with cervical cancer.<sup>(8,15)</sup> However, the various studies on *IFNG* polymorphisms and cervical cancers have shown conflicting results. For example, a study from South Africa revealed no significant differences between cervical cancer patients and controls.<sup>(13)</sup> The aim of our study was to investigate the association between the *IFNG*+874T>A polymorphism and cervical cancer.

## METHODS

### Research design

A case-control study was carried out from June 2010 to December 2010 in the gynecology outpatient clinic of Hasan Sadikin Hospital, Bandung, Indonesia.

### Research subjects

This study enrolled consecutive new female patients with cervical cancer (n=98), diagnosed by the gynecologists on duty as cervical carcinoma stage IIA/IIB, according to the Fédération Internationale de Gynécologie et

d'Obstétrique (FIGO) staging system. We limited enrollment to cervical cancer patients who presented to the clinic from June 2010 to December 2010. Excluded were women who were less than 18 years old, pregnant, previously treated with chemotherapy or radiation for any cancer, and also those lacking histological data. In the same period, consecutive females who came for PAP smears (n=81) were included as controls. The control group was not matched to the cervical cancer group for age and ethnicity. Both cervical cancer patients and controls were interviewed for recording purposes, using the standardized medical questionnaire in the clinic.

#### **DNA extraction and genotyping**

DNA was isolated from venous blood collected in ethylenediaminetetraacetic acid (EDTA) tubes according to the manufacturer's protocol (Qiagen Blood Mini Kit, Germantown, MD). Bi-allelic *IFNG*+874 T>A polymorphism was analyzed by a modification of the amplification refractory mutation system-polymerase chain reaction technique (ARMS – PCR). In brief, PCR was performed using combinations of primers consisting of a sense primer for allele A or T and a common antisense primer. To assess the success of PCR amplification in both reactions, an internal control was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (HGH). The PCR conditions were as follows: hold at 95°C for 1 min, 10 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 50 s, extension at 72°C for 40 s, followed by 20 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 50 s, and extension at 72°C for 50 s, and final extension at 72°C for 10 min then at 4°C for an indefinite time for short-term storage. The PCR products were analyzed by electrophoresis on 1.5% agarose gel, showing PCR products of human growth hormone (426 bp) as internal controls. Each individual showed the T and A alleles separately, identified by the 261 bp band.

#### **Ethical clearance**

Written consent was obtained from all subjects, and the study protocol was approved by the Institutional Review Board of Padjadjaran University, Bandung.

#### **Statistical Analyses**

The allelic and genotypic frequencies were obtained by direct counting. The Hardy-Weinberg equilibrium (HWE) of each polymorphism was checked using the program HWE. The program CONTING was used to calculate  $\chi^2$  and associated values for contingency tables. Data from the questionnaire and genotyping were analyzed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). All statistical analyses were two-sided and *P* values <0.05 were considered as statistically significant. The odds ratio (OR) was determined to calculate possible significant differences in genotypes.

#### **RESULTS**

Of the 98 subjects with cervical cancer, 29 had missing histological data, 5 had no malignant cells found in the samples (designated as “others” in Table 1), thus, only 64 participants were further enrolled (Table 1). Squamous cell carcinomas (SCC) were more prevalent in the study population (n = 53 of 64; 82.8%), of which most were non-keratinizing (n=43 of 53; 81.1%). The patient characteristics could not be described in this study, as some of the medical records were lacking in data on age, marital status, number of children, smoking status, and high risk sexual behavior. This caused difficulties in analyzing further data, therefore, the analysis was only based on the histopathological data.

In the control group the excluded subjects were as follows: 18 had missing histological data, 21 showed abnormal findings on cytological examination such as low-grade (n=16) and high-grade squamous intraepithelial lesions (n=5) (Table 1).

Tabell. Histopatological distribution of the subjects

Histopatological data	Cervical cancer n (%)	Control
Adenocarcinoma	9 (14.1)	
Adenosquamous cell carcinoma	2 (3.1)	
Squamous cell carcinoma	53 (82.8)	
Non-keratinizing	43	
Keratinizing	10	
Others	5*	
Missing data	29*	
HSIL		5*
LSIL		16*
NILM		42
Missing data		18*

Note: \*were not included in the study, this includes: others and missing data for cervical cancer patients and HSIL, LSIL and missing data for control group. Others designated for biopsies contained no abnormalities; HSIL= high-grade squamous intraepithelial lesion; PAP= papanicolau smear; LSIL=low-grade squamous intraepithelial lesion; NILM=negative intraepithelial lesions or malignancy

#### *IFNG* +874 T>A polymorphism

In the ARMS-PCR method using primer combinations, DNA samples were of good quality and positive controls using primers for human growth hormone (hGH) were shown in the gel electrophoresis (Figure 1). The genotypes of the *IFNG* +874 T>A polymorphism were in Hardy – Weinberg equilibrium in the total group of individuals as well as in the patient and control groups. The distribution of *IFNG* genotypes TT, TA and AA for women with cervical cancer who met the inclusion criteria (n=64) and had negative intraepithelial lesions or malignancy (NILM) (n = 42) were 14.1%, 50.0%, 35.9% and 7.1%, 52.4%, 40.5%, respectively, and no significant differences could be observed

between both groups (p=0.64) (Table 2). As the SCC group was the most prevalent in our study population, these cases were stratified further, to make the groups more homogenous. The distribution of *IFNG* genotypes for patients with SCC (n~53) was as follows: TT (15.1%), TA (54.7%), AA (30.2%), and no significant differences could be observed between the SCC group and NILM group (p=0.29) (data now shown).

#### DISCUSSION

The susceptibility to HPV infection that leads to cervical cancer may be influenced by variations in the *IFNG* gene that codes for the

Table 2. Distribution of allele and genotype *IFNG* T874A in cervical cancer and control

Polymorphism	allele / genotype	Cervical cancer n (%)	Control n (%)	p value
<i>IFN</i> +874 T/A	T	50 (39.1)	28 (33.3)	0.39
	A	78 (60.9)	56 (66.7)	
	TT	9 (14.1)	3 (7.1)	0.64
	TA	32 (50.0)	22 (52.4)	
	AA	23 (35.9)	17 (40.5)	

Note: NILM=negative intraepithelial lesion or malignancy

cytokine IFN- $\alpha$ . Our study showed no significant difference between cervical cancer patients and their controls with negative intraepithelial lesions (NILM). The distribution of the T and A allelic and genotypic frequencies in our study results is in concordance with the study in Brazil.<sup>(16)</sup> However, in contrast to the latter, we found no significant differences even after stratifying the patients into a more homogenous group e.g. only SCC and NILM. Interestingly, the frequency of the TT genotype, denoting high-level IFN- $\alpha$  producers, was lower than that of the AA genotype, i.e. the prevalence of low-level IFN- $\alpha$  producers in our population was similar to those in the Indian and Brazilian studies.<sup>(8,16)</sup> Ethnic background differences may play a role in the distribution of *IFNG* +874 genotypes.

Cervical cancer is the second most common cancer in women worldwide and persistent infection with high risk types of HPV, e.g. HPV 16 and HPV 18, are strongly associated with development of cervical cancer.<sup>(17)</sup> Although many women with normal cytology are also infected with high-risk types of HPV,<sup>(5)</sup> only few women will ever develop cervical cancer, as IFN- $\alpha$  production may influence HPV clearance and prevent malignant progression.<sup>(1)</sup> The majority of HPV infections are cleared without further consequences for the host and only a small percentage of women,

who are persistently infected with oncogenic genotypes, will develop cancer. It is suggested that IFN- $\alpha$  is crucial to control of HPV. IFN- $\alpha$  production and function are encoded by the *IFNG* gene, and the +874 A allele of *IFNG* may influence the secretion of the cytokine.<sup>(18)</sup>

A meta-analysis to determine the association of *IFNG* gene polymorphisms and various types of cancer showed that although in general the +874 T allele of *IFNG* showed no protective significant association, yet a strong association was shown with cervical cancer.<sup>15</sup> For example, in two studies at different sites in India, variation in the *IFNG* +874 AA genotype as a low IFN- $\alpha$  producer was associated with a significantly increased risk of cervical cancer.<sup>(8,19)</sup>

Clearly, studies in human viral diseases point out a complex immune system response in controlling the infection. IFN- $\alpha$  may not be the only cytokine influencing HPV clearance or cervical cancer development. Other Th2 cytokines such as IL-4<sup>(18)</sup> and IL-10<sup>(21,22)</sup> may also play a role in HPV clearance, e.g. the IL-10 -1082G polymorphism has been shown to be associated with clearance of HPV infection<sup>(21)</sup> and increased IL-10 production.<sup>(22)</sup> Other studies showed that interferon alpha-17 (*IFN17*) *Ile184Arg* polymorphism may also play a significant role.<sup>(23)</sup> Moreover, the tumor suppressor gene TP53 is associated with cervical cancer as a genetic co-factor.<sup>(24)</sup> However, a pooled analysis of studies on polymorphism in codon 72 of TP53 has indicated that that excessively high risks were most likely not due to clinical or biological factors, but to errors in study methods.<sup>(25)</sup> Better study methods and larger numbers of participants need to be underlined.

We acknowledge that our study has several limitations, among others missing data in medical records, such that age and ethnicity were not matched between cancer patients and their control group. Furthermore, the small number of cases and controls is another limitation of this study that has prevented us to come to any major



Figure 1. Electrophoresis of *IFNG* T874A polymorphisms

Upper band is an internal control using primer HGH (500bp). Every individual has 2 lanes, i.e. lane T and lane A. Person no. 1 has genotype TA, person no. 2 has genotype AA etc.

conclusions. The distribution of *IFNG* +874 T/A polymorphisms in our study population may not reflect that of cervical cancer in Indonesia *per se*; however, this study may provide direction for studies involving much larger groups of patients. *IFNG* genotyping in larger numbers of participants from the Indonesian archipelago with different ethnic backgrounds may also reveal more valuable information. Further understanding of the role of this cytokine may contribute to the development of a biomarker of HPV infection and result in the improvement of the treatment of squamous intraepithelial lesions.

## CONCLUSIONS

*IFNG* polymorphism at +874 T>A seems not to contribute to susceptibility to cervical cancer. Identification of other variants in *IFNG* gene signaling and its role in the development of cervical cancer need to be further examined to provide information on biological markers that would be useful for the development of diagnostic and therapeutic strategies.

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