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Prevalence of the *NKG2D* Thr72Ala Polymorphism in Patients with Cervical Carcinoma

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Background: The natural killer group 2, member D (NKG2D) receptor is mainly situated on the surface of NK and $CD8^+$ αβ T cells that are involved in the defense against viral agents and in cancer immunosurveillance. The G > A transition (Thr72Ala) (rs2255336) located in the *NKG2D* region encoding the transmembrane part of this receptor has been associated with decreased functionality of NK and T cells. *Methods:* Using polymerase chain reaction–restriction fragment length polymorphisms, we examined the *NKG2D* Thr72Ala polymorphism in patients with cervical cancer ($n = 353$) and controls ($n = 366$) in a Polish population. *Results:* We observed an increased frequency of Thr/Thr or/and Thr/Ala genotypes in controls compared with all patients with cervical cancer; however, these differences were not significant. We found a significantly increased frequency of the *NKG2D* 72Thr allele in controls than in all patients (odds ratio [OR] = 0.7410 [95% confidence intervals (CI) = 0.5683–0.9662, $p = 0.0265$]). Moreover, stratification of patients based on cancer stage showed a significant increase in the Thr/Thr genotype frequency (OR = 0.3086 [95% CI = 0.09097–1.047, $p = 0.0461$]), as well as in the Thr/Thr and Thr/Ala genotype frequency (OR = 0.4504 [95% CI = 0.2891–0.7018, $p = 0.0003$]), in controls compared with patients with cervical cancer in stages III and IV. The frequency of the *NKG2D* 72Thr allele was also significantly increased in controls as compared with patients in stage III and IV cancer (OR = 0.4699 [95% CI = 0.3170–0.6967, $p = 0.0001$]). *Conclusion:* Our studies may suggest that the women with cervical cancer bearing the *NKG2D* 72Thr gene variant might be protected against progression to advanced stages of this cancer.

Introduction

Cervical cancer is one of the most prevalent female malignant neoplasms and the main cause of early-onset death in women (Arbyn *et al.*, 2007). The human papilloma virus (HPV) is recognized as being the major cause of cervical tumors and their precursor lesions, which are of varying degrees and are called cervical intraepithelial neoplasias (CIN) (Walboomers *et al.*, 1999; Giuliano *et al.*, 2002). HPV is one of the common viruses sexually transmitted, existing in about 100 different subtypes that differ in genetic and oncogenic potential (Muñoz *et al.*, 2003). The oncogenic subtypes of HPV contributing to cervical carcinogenesis include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and others (Muñoz *et al.*, 2003). It has been shown that both adaptive and innate immune responses are involved in the clearance of HPV infection (Kobayashi *et al.*, 2004; Frazer, 2007; Stanley, 2008; Peng *et al.*, 2007). Spontaneous regression of HPV16-positive squamous intraepithelial lesions of the cervix has been accompanied by $CD4^+$ T-cell responses against the viral E7 peptide (Peng *et al.*, 2007). By contrast, in cervical tissue, the innate immune response acts against various viruses using the activation of toll-like receptors, as well as dendritic and natural killer (NK) cells (Wira *et al.*, 2005; Manickam *et al.*, 2007; Hasan *et al.*, 2007). NK cells spearhead the defense against viral agents and play an elementary role in immunosurveillance against various cancers (Lanier *et al.*, 2008; Waldhauer and Steinle, 2008). These cells are activated for the killing of cancer cells via complex interactions between various receptors, including natural killer group 2, member D (NKG2D), and ligands located on the surface of cancer cells (Sivori *et al.*, 1997; Pessino *et al.*, 1998; Pende *et al.*, 1999; Wu *et al.*, 1999; Moretta *et al.*, 2006). The NKG2D receptor is a member of the C-type lectin family (Houchins *et al.*, 1991). NKG2D is also involved in the adaptive immune response as a co-stimulatory receptor in $CD8^+$ αβ T cells and is found on γδ T cells (Wu *et al.*, 1999; Moretta *et al.*, 2006; Houchins *et al.*, 1991). The ligands of NKG2D encompass some of the cytomegalovirus UL16-binding proteins (ULBPs), and major histocompatibility complex (MHC) class I-related chains A (MICA) and B (MICB) (Raulet, 2003; Bahram *et al.*, 1994; Cosman *et al.*, 2001; Chalupny *et al.*, 2003; Bacon *et al.*,

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2004). Some studies have recently demonstrated a change in the number of NKG2D molecules on the surface of immune cells from patients with cervical cancer (Arreygue-Garcia *et al.*, 2008; Garcia-Iglesias *et al.*, 2009).

HPV infection alone is not sufficient for cervical tumorigenesis. The use of oral contraceptives, smoking, and genetic factors may also be involved in cervical tumor development (Castellsague *et al.*, 2003; Magnusson *et al.*, 2000). It has been reported that the G > A transition (Thr72Ala) (rs2255336) situated in the *NKG2D* region encoding the transmembrane part of this receptor may be associated with decreased functionality of NK and T cells (Kabalak *et al.*, 2010; Hayashi *et al.*, 2006). Therefore, we explored the genotype and allele frequencies of the *NKG2D* Thr72Ala polymorphism in patients with cervical cancer in a Polish population.

Patients and Methods

Patients and controls

The patient group consisted of three hundred fifty-three women with a histologically determined stage of cervical carcinoma according to the International Federation of Gynecology and Obstetrics. Patient data were collected for patients seen between April 2007 and July 2011 at the Department of Radiotherapy, Greater Poland Cancer Center in Poznań, Poland (Table 1). Three hundred sixty-six unrelated healthy women volunteers, of a similar age to the patients, were used as controls (Table 1). Women with cervical cancer and controls were Caucasians, collected from the Wielkopolska area of Poland. All subjects involved in the study provided written informed consent. The study was approved by the local ethics committee of the Poznań University of Medical Sciences.

Table 1. Clinical and Demographic Characteristics of Patients and Controls

Characteristic	*Patients (n = 353)*	*Controls (n = 366)*
[a]Mean age ± SD	49.7 ± 10.4	48 ± 9.9
Tumor stage		
IA	51 (14.4%)	
IB	54 (15.3%)	
IIA	44 (12.5%)	
IIB	51 (14.4%)	
IIIA	74 (20.9%)	
IIIB	61 (17.3%)	
IVA	3 (0.8%)	
IVB	15 (4.2%)	
Histological grade		
G1	81 (22.9%)	
G2	153 (43.3%)	
G3	52 (14.7%)	
Gx	67 (19.0%)	
Histological type		
Squamous Cell Carcinoma	331 (93.8%)	
Adenocarcinoma	14 (3.9%)	
Other	8 (2.3%)	
HPV genotypes		
16 and 18	225 (63.7%)	
16, 18, 31, 33, 35, 39,45,51,52,56,58,59 and 68	353 (100%)	

[a]age at first diagnosis.
HPV, human papilloma virus.

Genotyping

DNA was isolated from peripheral leucocytes by employing a standard salting-out procedure. The *NKG2D* Thr72Ala (c.214A > G) (rs2255336) polymorphic variant was identified by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). PCR was performed employing the primer pair 5′ GAGGTATTTATGTTCTGTTCTGG 3′and 5′ TCTACTTCTCTGTTGTCACTTAC 3′. The PCR-amplified fragments of *NKG2D* that were 271bp in length were isolated and subjected to digestion by endonuclease MaeIII (/GTNAC) Roche Diagnostic GmbH, (Penzberg, Germany). The *NKG2D* 72Ala allele remained uncut, whereas the *NKG2D* 72Thr allele was cleaved into 217bp and 54bp fragments. The digestion products were separated by electrophoresis on 3% agarose gel. The *NKG2D* Thr72Ala polymorphism was confirmed by repeated PCR-RFLP and commercial sequencing analysis.

Statistical analysis

The distribution of genotypes in patients and controls was examined for deviation from the Hardy–Weinberg equilibrium. The chi-squared (χ^2) test or Fisher exact test was used to determine the differences in genotypic and allelic distribution between patients and controls, and a p-value < 0.05 was considered statistically significant. The odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. Moreover, the polymorphism was tested for association with cervical cancer incidence using the χ^2-test for trend (p_{trend}). Bonferroni correction for multiple comparisons was used, and both p-values, before (p) and after correction (p_{corr}), were determined. Power calculations for this study were performed using Quanto software (Gauderman WJ, Morrison JM. QUANTO 1.2: A computer program for power and sample size calculations for genetic-epidemiology studies, URL: http://hydra.usc.edu/gxe, 2006).

Results

Distribution of NKG2D Thr72Ala genotypes and alleles in women with cervical cancer in stage I, II, III, and IV

Analysis of the *NKG2D* Thr72Ala polymorphism did not reveal a significant deviation from the Hardy–Weinberg equilibrium in all women with cervical cancer and controls.

The frequency of the *NKG2D* Thr/Thr genotype in controls and all women with cervical cancer was 0.06 and 0.03, respectively (Table 2). The distribution of the heterozygous genotype frequency was approximately 1.2-fold higher in controls than in women with cervical cancer and was 0.31 and 0.27, respectively (Table 2). We observed an increased frequency of the Thr/Thr genotype in controls as compared with all patients with cervical cancer; however, these differences were not significant (OR = 0.5029 [95% CI = 0.2401–1.053, $p = 0.0637$]). There was also an increased frequency of the Thr/Thr and Thr/Ala genotypes in controls compared with all patients, but these differences were also not significant (OR = 0.7531 [95% CI = 0.5517–1.028, $p = 0.0736$]) (Table 2). We found a significantly higher frequency of the *NKG2D* 72Thr allele in controls than in patients (OR = 0.7410 [95%

Table 2. Prevalence of *NKG2D* Thr72Ala Polymorphisms Among Patients with Cervical Cancer and Healthy Individuals

	Genotype distribution (frequency)			*Minor allele frequency*	*Odds Ratio (95% CI)*	*p*	p_{trend}
n	Ala/Ala	Ala/Thr	Thr/Thr				
Controls *n* = 366	232 (0.63)	112 (0.31)	22 (0.06)	0.21			
Cervical cancer in stage I, II, III and IV *n* = 353	246 (0.70)	96 (0.27)	11 (0.03)	0.17	0.5029 (0.2401–1.053)[a]	0.0637[d]	0.0313
					0.7531 (0.5517–1.028)[b]	0.0736[d]	
					0.7410 (0.5683–0.9662)[c]	0.0265[d]	
Cervical cancer in stage III and IV *n* = 155	123 (0.79)	29 (0.19)	(0.02)	0.11	0.3086 (0.09097–1.047)[a]	0.0461[e]	0.0003
					0.4504 (0.2891–0.7018)[b]	0.0003[d]	
					0.4699 (0.3170–0.6967) [c]	0.0001[d]	

The Odds Ratio (OR) was calculated for patients [a](Thr/Thr genotype vs Ala/Thr and Ala/Ala enotypes).
[b](Thr/Thr and Ala/Thr genotype vs Ala/Ala genotype). We also determined the OR for the risk allele.
[c](Thr vs Ala allele).
[d]uncorrected Chi^2.
[e]Fisher exact test.
CI, confidence interval.

CI = 0.5683–0.9662, $p = 0.0265$]) (Table 2). The *p*-value of the χ^2-test for the trend observed for the *NKG2D* Thr72Ala polymorphism was also statistically significant ($p_{trend} = 0.0313$). Moreover, we observed a statistical difference in the genotype distribution between patients with different tumor stage $p = 0.0038$ ($p_{corr} = 0.0076$) (Table 3). A power analysis of this patient group predicted sufficient power to detect an association of the *NKG2D* Thr72Ala polymorphism with a genetic effect of 0.5 or less for the Thr/Thr and Ala/Thr vs Ala/Ala genotypes or a genetic effect of 0.1 and less for the Thr/Thr vs Thr/Ala and Ala/Ala genotypes (Table 4).

Distribution of NKG2D Thr72Ala genotypes and alleles in women with cervical cancer in stage III and IV

The prevalence of the homozygous Thr/Thr genotype was approximately threefold higher in controls than in women with cervical cancer in stage III and IV (Table 2). The frequency of the *NKG2D* Thr/Ala heterozygous genotype in women with cervical cancer in stage III and IV was 0.19 (Table 2). We found a significantly increased frequency of the Thr/Thr genotype in controls compared with patients with cervical cancer in stage III and IV (OR = 0.3086 [95% CI = 0.09097–1.047, $p = 0.0461$]). There was also a statistically increased frequency of the Thr/Thr and Thr/Ala genotypes in controls compared with these patients (OR = 0.4504 [95% CI = 0.2891–0.7018, $p = 0.0003$]) (Table 2). We also observed a significantly higher frequency of the *NKG2D* 72Thr allele in controls than in this patient group [OR = 0.4699 (95% CI = 0.3170–0.6967, $p = 0.0001$) (Table 2)]. The *p*-value of the χ^2-test for the trend observed for the *NKG2D* Thr72Ala polymorphism was also statistically significant ($p_{trend} = 0.0003$).

A power analysis of this patient group predicted sufficient power to detect an association of the *NKG2D* Thr72Ala polymorphism with a genetic effect of 0.5 or less for the Thr/Thr and Thr/Ala vs Ala/Ala genotypes but not for the Thr/Thr vs Ala/Thr and Ala/Ala genotypes (Table 4).

Table 3. Prevalence of *NKG2D* Thr72Ala Genotypes Differentiated by Patient Tumor Stage and Histological Grade

	Tumor stage				*Histological grade*		
	I	*II*	*III*	*IV*	*G1*	*G2*	*G3*
NKG2D Thr72Ala							
Ala/Ala	70	53	110	13	48	116	39
Ala/Thr	29	38	25	4	30	33	11
Thr/Thr	6	2	2	1	3	4	2
				$p = 0.0038$ ($p_{corr} = 0.0076$)			$p = 0.0940$

Data are presented as number of patients; *p*-values represent the significance of genotype distribution between tumor characteristics and were determined by the chi-squared test.

Discussion

NKG2D is one of the best recognized activating receptors of the NK cells that interacts with MICA, MICB, or the ULBPs to possibly trigger the killing of malignant cells (Diefenbach

Table 4. Power Analysis

	Genetic model	
Genetic effect	*Thr/Thr vs Ala/Thr and Ala/Ala*	*Thr/Thr and Thr/Ala vs Ala/Ala*
Cervical cancer in stage I, II, III, and IV (*n* = 353)		
0.1	0.9599	0.9999
0.3	0.7089	0.9999
0.5	0.3706	0.9885
0.7	0.1480	0.6159
0.9	0.0592	0.1043
Cervical cancer in stage III and IV (*n* = 155)		
0.1	0.7832	0.9999
0.3	0.4732	0.9995
0.5	0.2376	0.9047
0.7	0.1074	0.4165
0.9	0.0556	0.0826

Power analysis was conducted with the following parameters: allele frequency 0.21, disease population frequency 0.018, and significance level 0.05, two-sided.

et al., *Nature* 2001; Pende *et al.*, 2002; Carbone *et al.*, 2005). The down-regulation of NKG2D in NK and T cells by metalloprotease-mediated release of MICA and MICB ligands from the cell surface has been demonstrated in various cancers (Jinushi *et al.*, 2005; Holdenrieder *et al.*, 2006; Salih *et al.*, 2006; Groh *et al.*, 2002). The activating ligands of NKG2D were also differentially expressed during the progression to cervical cancer (Textor *et al.*, 2008). Arreygue-Garcia *et al.* (2008) have reported a reduced number of NKG2D receptors on the surface of NK and T cells in women with both cervical cancer and CIN (Arreygue-Garcia *et al.*, 2008). Moreover, in patients with cervical cancer, the down-regulation of NKG2D was linked to low NK cell activity along with increased HPV16 infection and cervical tumor progression (Garcia-Iglesias *et al.*, 2009).

We observed a statistically significant trend for the *NKG2D* Thr72Ala polymorphism in all patients with cervical cancer. Moreover, our study showed statistically significant differences in genotype distribution between patients with different tumor stages. Specifically, we found a statistically significant lower frequency of the *NKG2D* 72Thr gene variant in women with cervical cancer in stage III and IV as compared with healthy women.

This suggests that the *NKG2D* 72Thr gene variant may protect against cervical tumor progression by modulating cytotoxic T and NK cells. Hayashi *et al.* (2006) demonstrated that cancer patients having the *NKG2D* Ala/Ala genotype displayed decreased cytotoxicity of NK cells (Hayashi *et al.*, 2006). Moreover, Kabalak *et al.* (2010) used anti-CD3 and anti-NKG2D antibodies to demonstrate decreased proliferation of peripheral blood lymphocytes bearing the *NKG2D* 72Ala/Ala genotype than lymphocytes with the *NKG2D* 72Thr gene variant (Kabalak *et al.*, 2010). They also demonstrated that the *NKG2D* 72Thr gene variant could be a protective factor in the development of systemic lupus erythematosus (Kabalak *et al.*, 2010).

The *NKG2D* Thr72Ala substitution is situated close to the transmembrane arginine at position 66, which binds via hydrogen bonds with the DAP10 adapter molecule (Garrity *et al.*, 2005). The placement of this substitution may produce altered intracellular signals after ligand binding to the NKG2D molecule. A possible role of DAP10 adapter molecule levels in the modulation of NK cytotoxicity in cancer patients has also been reported (Hyka-Nouspikel and Phillips, 2006). Hyka-Nouspikel *et al.* (2007), using the murine model, reported that DAP10 signaling is involved in adjustments in the activation threshold controlling host immunity against tumors (Hyka-Nouspikel *et al.*, 2007). Recently, Lee *et al.* (2011) demonstrated that reduced NK cytotoxicity in cancer patients due to down-regulation of surface NKG2D resulted from reduced levels of DAP10 molecules (Lee *et al.*, 2011).

Our study may suggest that cervical cancer patients bearing the *NKG2D* 72Thr gene variant might be protected against the progression to advanced stages of this cancer. However, to confirm the role of the *NKG2D* Thr72Ala polymorphism in cervical cancer, this study should be replicated in a larger and independent cohort, and functional studies should be conducted on this polymorphism.

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