Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Human leukocyte antigen *(HLA)-G* gene polymorphism in patients with non-small cell lung cancer

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Keywords
HLA-G; lung cancer; promoter; single nucleotide polymorphism.

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Received: 27 September 2014;
Accepted: 22 December 2014.

doi: 10.1111/1759-7714.12232

Thoracic Cancer **6** (2015) 613–619

Abstract

Background: Lung cancer represents the highest morbidity and mortality caused by neoplasms in the world; therefore researchers continue to search for new tools to diagnose and treat the disease. The aim of the study was to establish the role of single nucleotide polymorphisms (SNP) in the promoter region of the human leukocyte antigen (HLA)-G gene in patients with non-small cell lung cancer.
Methods: We enrolled 143 patients with a mean age of 63 years, diagnosed with non-small cell lung cancer, in the study. Adenocarcinomas made up 33% of the cases. Patients in stage III or IV of the tumor node metastasis staging system made up 59%. Two polymorphic sites in the promoter region of the HLA-G gene were genotyped (−725C>G>T and −716T>G).
Results: All genotyped SNPs were in Hardy-Weinberg equilibrium. No proof of a relationship between genotype −725C>G>T or −716T>G and the risk of lung cancer compared with healthy volunteers from the literature was found. We also found no correlation between the two SNPs and survival time, histological type of cancer, T stage, the presence of remote metastases or performance status according to the Eastern Cooperative Oncology Group (ECOG) scale. The only association we found was genotype −725C>G>T and the degree of lymph node metastases (N stage).
Conclusions: SNPs of the promoter of the HLA-G gene may have an impact on the development of lymph node metastases. In the study we did not prove a relationship between the examined SNPs and the course of the disease because of the small patient groups studied.

Introduction

According to available statistics, lung cancer is the most frequently recognized neoplasm in the world.[1,2] Symptoms of the disease usually appear in the late stage, which results in poor prognosis.

There are two main types of lung cancer, which differ in clinical course and susceptibility to treatment and prognosis: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is more frequent and is subdivided into squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and a few other less frequent subtypes.[3,4] Diagnosis is based on cytological and histological examination of different specimen biopsies.[5] Treatment of lung cancer consists of thoracic surgery procedures, chemotherapy, radiotherapy, and combinations of these methods.[6–9] However, the efficacy of these therapies remains poor.

The aetiology of lung cancer is not yet fully known. According to the cancer immunoediting hypothesis, supervision of the immune system may be crucial to eliminate tumor cells until the escape phase.[10–12] There are many mechanisms which take part in cancer immunosurveillance, but cancer cells also develop different mechanisms to escape from elimination or equilibrium phases.[13,14] One of them may be a change in HLA (human leukocyte antigen) molecule expression on the cancer cell surface.[15,16] The main role of HLA class I molecules is to present autoantigens to cytotoxic T lymphocytes, which protect the cells against lymphocyte activation and also against NK cell reaction. HLA-G belongs to the non-classical major histocompatibility complex class I molecules, which are expressed only in immunoprivileged tissues, such as the fetomaternal barrier.[17–19] Because of its expression, the cytotrophoblast is protected against maternal immunological immunocytotoxity.

One of the mechanisms of the escape phase revealed by cancer cells is the loss of HLA molecules to become invisible for T lymphocytes. But this leads to an NK cell reaction. HLA-G expression on cancer cells is the mechanism of specific immune tolerance function that makes them resistant to cytotoxicity of NK cells and cytotoxic T lymphocytes.[20,21] There is some evidence that HLA-G expression takes part in the pathology of different neoplasms, including lung cancer.[22,23] Increased expression of HLA-G has been found in many different solid tumors.[24,25]

The HLA-G gene polymorphism is very limited.[26,27] Polymorphism of the promoter region may influence HLA-G levels caused by different affinity of transcriptional and post-transcriptional factors.[28] To date, 29 SNPs in the promoter region of the HLA-G gene have been reported.[29]

The aim of the study was to establish the role of HLA-G gene polymorphism in patients with NSCLC. We analysed two single nucleotide polymorphisms (SNPs) in the promoter region of the HLA-G gene, −725C>G>T and −716T>G, which may have an influence on gene expression.

Materials and methods

Patient data

One hundred and forty-three patients diagnosed with NSCLC were enrolled in the study. They were hospitalized in the Department of Pulmonology and Lung Cancer, Medical University of Wrocław, in 2010–2012. Diagnosis was based on cytological or histological examination. Patients were divided into groups according to the World Health Organization classification of tumors (2004) and the tumor node metastasis (TNM) staging system (7th edition).[30] Clinical characteristics of patients included the Eastern Cooperative Oncology Group scale and history of smoking.[31] Blood samples were collected before any oncological therapies. Patients were treated with chemotherapy, radiotherapy or surgery according to recommendations. We observed the response to the treatment and the course of the disease. The treatment response was mainly based on radiological examinations including chest computed tomography (CT, Response Evaluation Criteria in Solid Tumors), chest X-rays, if chest CTs were not available, and clinical data, such as the appearance of remote metastases (central nervous system, bones, adrenal glands). Lymphangitis carcinomatosa, pleural effusion, infiltration of thorax tissues or metastases to the other lung resulted from progression of the disease.

Polymerase chain reaction genotyping

Genetic examinations were performed at the Laboratory of Immunogenetics, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław. There were four stages of the molecular procedure: genomic DNA extraction from whole blood leucocytes (Invisorb Spin Blood Midi Kit, Protocol 2, Strateg Molecular, Stratec Biomedical AG, Birkenfeld, Germany); spectrophotometric measurement of concentrations and purity of DNA preparations; amplification of the fragments of the HLA-G gene promoter region containing analysed SNPs (polymerase chain reaction, PCR); and temperature gradient gel electrophoresis (TGGE).

Table 1 Histological types of lung cancer in the study group and the stage of advancement according to TNM

Histological type	Stage		I	II	III	IV
Adenocarcinoma	n	48	16	10	9	13
	%	33.6	33.3	20.8	18.8	27.1
Squamous cell	n	42†	7	11	12	11
	%	29.4	17.1	26.8	29.3	26.8
Large cell	n	14	6	3	4	1
	%	9.8	42.9	21.4	28.6	7.1
NOS	n	39	4	1	15	19
	%	27.3	10.3	2.6	38.5	48.7
Total	n	143	33	25	40	44
	%	100%	23.2	17.6	28.2	31

†One person with the squamous cell carcinoma the stage was not defined. NOS, not otherwise specified; TNM, tumor node metastasis.

Electrophoresis

Temperature gradient gel electrophoresis was used to obtain different band patterns of the HLA-G gene promoter fragment. This method enables diagnosis of SNPs within DNA fragments of the same length because the presence of SNP changes the denaturation temperature of the DNA strand in the gel.

We compared the results of our group of patients with results published by Wiśniewski *et al*.[32] In their study, a group of healthy controls (288 volunteers) were diagnosed using the same method by the same researcher. The control cohort was ethnically matched (Polish caucasians) and consisted of 171 women and 117 men; the mean age (for 164 individuals, for whom this information was available) was 31.3 ± 9.8 years.

The Bioethics Committee of the Medical University of Wrocław approved the study. Informed consent was obtained from the included patients.

Statistical analysis

The median was used as the location parameter. Additionally, first and third quartiles and minimal and maximal observations were reported. Time to death was analysed with the proportional hazards model and survivor function was estimated with the Kaplan-Meier curve. Departure from the Hardy-Weinberg equilibrium was tested with the Chi-

Table 2 Distribution of genotypes and allele frequencies of –725C>G>T of the *HLA-G* gene in patients with lung cancer

Genotyes	CC	CG	CT	GG	GT	TT	Total
n	97	34	7	2	3	0	143
%	67.83	23.78	4.9	1.4	2.1	0	100%
Alleles	C	G	T	Total			
n	235	41	10	286	Hardy-Weinberg principle calculation		
%	82.17	14.34	3.5	100%	$\chi^2_{ss=3} = 2.372$		
					$P = 0.7528$*		

**P*- Monte Carlo method. HLA, human leukocyte antigen.

squared test. In the case of small numbers, distribution of the test statistics was estimated numerically. Odds ratio (OR) was computed as the measure of effect size. When it was reasonable, we assumed a log additive model of association between genotypes and clinical variables. Row effect, column effect, and row-column effect models were used to test associations in the two-way contingency tables. Analysis was performed with the R.2.14 platform (R Foundation for Statistical Computing, Vienna, Austria).

Results

Among 143 patients there were 33 women and 110 men of mean age 63 ± 6 years. The most frequently diagnosed histological type was adenocarcinoma (33%) (Table 1). Squamous cell carcinoma represented 30% of the diagnoses and large cell carcinoma 10%, but 27% remained not otherwise specified carcinomas. Thirty-one percent of the patients were in stage IV of the disease and 28% in stage III according to the four-stage system of cancer progression, based on TNM.

Seventy-five patients (52.4%) underwent surgery, with 53 of these patients subsequently treated with chemotherapy and/or radiotherapy. In 18 patients, only behavioral therapy was applied. Most of the patients treated with chemotherapy (41.5%) stabilized (the effect was observed during and at the end of chemotherapy), and 41.1% of operated patients had no relapse (during the time of observation). However, in 61 patients, the disease course was not known. During the observation period, 51 patients died, and the median survival time was six months.

At the genotype level: for rs1233334 (–725C>G>T) in Polish caucasians (N = 288), we found 70.5% C/C, 25.0% C/G, 1.1% G/G, 3.1% C/T, and 0.3% G/T. In the SNP database, we found 85.0% C/C and 15.0% C/G; other genotypes were not detected in caucasians (N = 60 only) by the method used. There was no information on the distribution of –716T>G (rs2249863) in caucasians in the database.

All genotyped SNPs were in Hardy-Weinberg equilibrium (Tables 2, 3), which means that analysed polymorphic sites were not under any disrupting factors, especially those of natural selection. On the other hand, new alleles are of low

Table 3 Distribution of genotypes and allele frequencies of –716 T > G of the *HLA-G* gene in patients with lung cancer

Genotypes	TT	GT	GG	Total
n	43	70	30	143
%	30.07	48.95	20.98	100%
Alleles	T	G	Total	
n	156	130	286	H-W
%	54.55	45.45	100%	$\chi^2_{ss=1} = 0.023$
				$P = 0.8782$

HLA, human leukocyte antigen.

Table 4 Distribution of genotypes frequencies of –725C>G>T of the *HLA-G* gene in patients with lung cancer and controls

Group	C alleles in genotype Genotype	0 GT or GG	1 CT or CG	2 CC	Total
Patients	n	5	41	97	143
	%	3.5	28.67	67.83	100%
Control	n	4	81	203	288
	%	1.39	28.13	70.49	100%
OR		2.62	1.06	1†	–
CI 95% dla OR		0.69;9.96	0.68; 4.09	–	–
OR total = 1.23, CI95 (0.83; 1.85)					
$\chi^2_{ss=1} = 0.83$, $P = 0.3623$					

†Homozygotes CC as reference group. CI, confidence interval; HLA, human leukocyte antigen; OR, odds ratio.

Table 5 Distribution of genotypes frequencies of –716 T > G of *HLA-G* gene in patients with lung cancer and controls

Group	T alleles in genotype Genotype	0 GG	1 GT	2 TT	Total
Patients	n	30	70	43	143
	%	20.98	48.95	30.07	100
Control	n	65	153	70	288
	%	22.57	53.13	24.31	100
OR		0.75	0.74	1†	–
CI 95% dla OR		0.42;1.34	0.46;1.19	–	–
OR total = 0.83, CI95 (0.61; 1.11) $\chi 2ss = 1 = 1.075$, $P = 0.2998$					

†Homozygotes TT as reference group. CI, confidence interval; OR, odds ratio.

Table 6 Lymph nodes metastases correlation with genotype –725C>G>T

Lymph node metastases†	N0		N1		N2		N3		Total	
–725C>G>T	n	%	n	%	n	%	n	%	n	%
CC	31	34.4	12	13.3	33	36.7	14	15.6	90	100%
CG	20	58.8	5	14.7	5	14.7	4	11.8	34	100%
CT	4	57.1	1	14.3	2	28.6	0	0	7	100%
GG	0	0	1	50	0	0	1	50	2	100%
GT	2	66.7	0	0	1	33.3	0	0	3	100%
Razem	57	41.9	19	14	41	30.1	19	14	136	100%
$\beta_{effect\ size} = 0.3168$ CI 95%(0.019; 0.71)	$\chi^2_{ss=1lrce} = 4.6$, $P = 0.0319$									

†n = 7 of Nx diagnosis were not included. CI, confidence interval.

frequencies, and even if natural selection acts against them, it is too difficult to detect within such a small patient sample. At the genotype level, the frequencies of the –725CC and –716GT genotypes were the highest. Neither the –725C>G>T nor the –716T>G genotype predisposes to lung cancer ($P = 0.7528$ and $P = 0.2998$, respectively).

No proof of a relationship between the genotype –725C>G>T or –716T>G and the risk of lung cancer compared with healthy volunteers from the literature was found (Tables 4, 5). The group of patients did not differ from the control group ($P = 0.3623$). Table 4 shows that heterozygotes with one C allele (CG or CT) at the –725 site have the same risk of developing the disease as homozygotes with CC alleles (OR = 1.06). Patients without C alleles (GT or GG) have twice as high a risk of becoming ill compared to CC patients (OR = 2.62), but we have no proof that the result is not a random sampling error (confidence interval [CI] 95% for OR 0.69; 9.96). We did not find a correlation between the two SNPs and survival time. We noted that patients with genotype CC at the –725 site had a 13% lower risk of death than CG and CT patients and 29% lower than GG and GT patients, but the result may be accidental ($P = 0.6998$). There was no evidence of any relationship between the SNPs and the histological type of cancer. There was also no impact of the examined genotypes on the T and M stages of the TNM scale. What we found was the correlation of genotype –725C>G>T and the extent of lymph node metastases: more C alleles in the genotype lead to a more advanced N stage ($P = 0.0319$) (Table 6). The risk that a patient with more C alleles than another patient also has more advanced lymph node metastases was 37% higher. There was no such correlation with genotype –716T>G. We also noted a tendency of a poorer response to chemotherapy in patients with more C alleles at the –725 position and patients with more T alleles at the –716 position (Tables 7, 8). Together with more C alleles at the –725 site, the frequency of partial regression decreases and increases the probability of stabilization and progression. If a patient with GT at the –716 site reacted with partial regression to chemo-

Table 7 The answer to systemic chemotherapy according to genotype –725C>G>T

–725C>G>T	Partial regression	Stabilization	Progression	Total
CC	8	12	9	29
CG	3	3	3	9
CT	1	1	0	2
GG	0	1	0	1
GT	0	0	0	0
Total	12	17	12	41†
$\beta_{effect\ size} = 0.21$; CI 95% (–0.69; 1.11) $\chi^2_{ss=1lrce} = 0.209$, $P = 0.6472$				

†n = 8 patients the results of chemotherapy were not known. CI, confidence interval.

Table 8 The answer to the systemic chemotherapy according to genotype –716T>G

–716T>G		Partial regression	Stabilization	Progression	Total
TT	n	3	6	5	14
	%	21.4	42.9	35.7	100%
GT	n	5	7	6	18
	%	27.8	38.9	33.3	100%
GG	n	4	4	1	9
	%	44.4	44.4	11.1	100%
Total	n	12	17	12	41†
	%	29.3	41.5	29.3	100%

$\beta_{effect\ size} = 0.39$; CI 95% (–0.17; 0.96)
$\chi^2_{ss=1lrce} = 1.94$, $P = 0.1635$

†n = 8 patients the results of chemotherapy were not known. CI, confidence interval.

therapy, then the homozygous TT patient has an OR 1.48 times higher chance of reacting with stabilization or progression. However, the 95% CI shows that we cannot exclude that there is no such dependence. The problem here concerns small groups of patients.

Discussion

Theoretically, each tissue may express HLA-G, and the nucleotide polymorphism of the gene may influence regulation mechanisms.[27,29] Studies on the variability of the HLA-G gene's promoter proved that the level of HLA-G gene transcription and HLA-G expression could be affected by SNPs.[33,34] We focused on the –725C>G>T SNP, because it is located close to the interferon regulatory factor-1 binding sequence and has already been found to be associated with several diseases, such as multiple sclerosis,[32] end-stage renal disease,[35] and sporadic miscarriage.[36] Its G allele creates a potential methylation site. As we know, the polymorphic sites –725C>G>T and –716T>G are near the interferon-specific regulatory element at the –744 site, which binds interferon response factor 1.[29] Polymorphisms may, thus, have an impact on the regulation of gene expression.[34,37] The examined SNPs may affect the activity of the promoter by influencing the binding place, which may lead to HLA-G expression.[38]

We also checked –716T>G simply because it was located on the same PCR product and influenced the migration in TGGE. The TGGE technique was chosen as the only method (apart from sequencing, which is more expensive) that differentiates between –725 alleles C, G, and T (the T allele was omitted in other studies).

In 2001, Urosevic *et al*. described messenger ribonucleic acid of HLA-G in lung cancer cells and lymphocytes, macrophages, and dendritic cells infiltrating the tumor, which was correlated with advanced stages of the disease and poor prognosis.[39] Subsequent studies showed HLA-G expression in NSCLC cells, which also correlated with lymph node metastases.[40,41] HLA-G expression may then be an independent prognostic marker of the disease course.

Our study focused on polymorphism of the promoter of the HLA-G gene in patients diagnosed with lung cancer. Patients were diagnosed and treated according to the recommendations. The study revealed no relationship between described SNPs and lung cancer morbidity and mortality. The only statistically significant correlation concerned the extent of metastases to lymph nodes: we noted a more advanced N stage in patients with more C alleles at the –725 site. This fact may confirm the role of HLA-G expression as prognostic marker. Other correlations did not reach the level of statistical significance and may need a larger sample size. According to our knowledge there have been no previous reports in the literature relating to the SNPs we examined: other *HLA-G* gene SNPs in lung cancer.

Conclusion

SNPs of the promoter of the *HLA-G* gene may have an impact on the development of lymph node metastases.

Disclosure

No authors report any conflict of interest.

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