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Genetic variants in the tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) do not contribute but Death Receptor (DR4) genes may contribute to susceptibility to head and neck cancer in Pakistani population

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Abstract

TRAIL mediated signaling in cancer cells has emerged as one amongst the most deeply studied molecular phenomenon. Recent breakthroughs have shown that overexpression of anti-apoptotic proteins, inactivation of pro-apoptotic proteins, transcriptional downregulation of TRAIL, DR4/DR5, degradation of DR/DR5 are some of the mechanisms which dramatically abrogate TRAIL induced apoptosis in cancer cells. Data obtained through genetic studies has highlighted highly polymorphic nature of DR4 and in accordance with this concept, we investigated the association between Head and Neck Cancer and polymorphisms in TRAIL (1595 C/T) and DR4 (C626G and A1322G) gene. We selected 100 patients with Head and Neck Cancer and 100 healthy, sex and age matched volunteers randomly. C626G and A1322G in DR4 gene were analyzed using Polymerase Change Reaction (PCR) – Restriction Fragment Length Polymorphism (RFLP) and Amplification Refractory Mutation System (ARMS) techniques respectively. For TRAIL gene 1595 C>T genotypes, there was no statistically significant role of homozygous CC or TT in Head and Neck cancer. CC was 58% in patients and 49% in controls. CT was 30% in patients and 43% in controls. TT was 12% in patients and 8% in controls. Allele frequency for C was noted to be 0.73 (patients) and 0.705 (controls), *p*-value (1). For T, 0.025 (patients) and 0.001(controls), *p*-value (0.88). The genotyping for DR4 gene 626 C>G polymorphism was done for 100 head and neck cancer patients and 100 age and sex matched healthy controls. All the genotypes for the polymorphism were in Hardy-Weinberg Equilibrium. For DR4626 C>G genotype, CC was 10% in patients and 2% in controls. GC was 63% in patients and 40% in controls. GG was 27% in patients and 58% in controls. Interestingly, in DR4 genotyping, CC was predisposing factor and GG acted as a protective factor. Allele frequency for C was noted to be 0.41 (patients) and 0.22 (controls), p-value (0.81). For G, 0.585 (patients) and 0.78 (controls), p-value (0.867). For the A1322G polymorphism, TT was 23% in patients and 36% in controls with a p-value 0.09 (table 6). CT was statistically significant in patients (45%) and controls (28%), p-value 0.04. CC was non-significant in patients (32%) and controls (36%), p-value 0.62 (table 6). C allele was 0.45% in patients and 0.5% in controls. T allele was 0.54% in patients and 0.5% in controls. Future studies must converge on somatic mutations, epigenetic mutations and expression analysis of TRAIL and DR4 to get a step closer to individualized medicine.

Key words: TRAIL, Cancer, Signaling, Apoptosis, Drug Resistance, DR4.

Introduction

Head and Neck Cancer (HNC) is a multifaceted and genomically complex disease as evidenced by rapidly accumulating preclinical and clinical studies. Data obtained through high-throughput technologies has considerably broadened field of molecular oncology and it is now clear that genetic/epigenetic mutations, inactivation of tumor suppressors, overexpression of oncogenes and loss of apoptotic cell death are some of the widely studied biochemical mechanisms. Apoptotic cell death is a comprehensively studied molecular mechanism and different proteins have been reported to effectively trigger apoptosis (1,2,3). TRAIL has received considerable appreciation because of its ability to selectively induce apoptosis in cancer cells while leaving normal cells intact. TRAIL transduced signals intracellularly through death receptor to induce apoptosis. Characteristically two different pathways have been noted to be functional in cancer cells to induce apoptosis. Death inducing signaling complex (DISC) is formed at death receptor by assembly of FADD and pro-caspase-8. Functionally active caspase-8 further activated caspase-3 to trigger downstream cellular processes (extrinsic pathway). Intrinsic pathway is functionalized via entry of truncated Bid into mitochondrion (2,3). Entry of tBid into mitochondrion promoted release of cytochrome c into cytoplasm. Cytochrome c, pro-caspase-9 and APAF assemble to form a signalosome which is noted to be essential for activation of caspase-9 (4,5).

Genetic studies have provided sufficient evidence of polymorphic nature of DR4 gene, but the most frequently noted polymorphism C626G is present within DR4 ectodomain. In this study we studied polymorphism in TRAIL and DR4 in HNC patients.

Materials and Methods

Sampling and DNA extraction

We selected 100 patients with HNC and 100 healthy, sex and age matched volunteers randomly. Histopathologically, most frequently registered type was squamous cell carcinoma (38%). stage 1 was 10%, stage 2 (23%), stage 3 (35%) and Stage 4 frequency was 30 % in HNC patients (Table 1). We used organic method to extract DNA from blood.

Table 1. Clinical and Histopathological history of patients.

Parameter	HNC (N=100)	
Age	49.51±13.654	
Age Group	Below 35	18
	Above 35	82
Stage	0	2
	1	10
	2	23
	3	35
	4	30
Gender	Male	51
	Female	49
Marital Status	Married	90
	Unmarried	10
Type Of Cancer	Oral	58
	Larynx	5
	Trachea	2
	Nasopharynx	14
	Oropharynx	14
	Hypopharynx	7
Histopathology	Eving sarcoma	3
	Infiltrating (SCC)	4
	Kreatinizing (SCC)	15
	Metastatic (SCC)	6
	Moderately Differentiated (SCC)	13
	Nasopharyngeal Carcinoma	2
	Non Kreatinizing (SCC)	6
	Sinonasal Adenocarcinoma	2
	Squamous Cell Carcinoma	38
	Undifferentiated (SCC)	1
	Undifferentiated Nasopharyngeal Carcinoma	2
	Well Differentiated (SCC)	8

PCR-RFLP Methods for TRAIL 1595C/T Polymorphism

Polymorphism was studied using specific set of primers consisting of Forward: 5′-TGA GCA CTA CAG CAA ACA TGA-3′ and reverse primer 5′-GCA CCA CTA AAA GAT CGC AGT-3′. 391-bp product was obtained after PCR and subsequently digested by RsaI (restriction enzyme) at 37°C. RsaI mediated digestion produced specific banding pattern. CC genotype was identifiable by detection of 332 and 59 bp. TT genotype was identifiable by detection of 186, 146, 59 bp fragments. Visualization of digested products was done under UV light after running on 2% agarose gel electrophoresis.

PCR-RFLP Methods for DR4 C626G Polymorphism

PCR conditions were optimized at 95 °C for 5 min, followed by 95 °C for 45 seconds, 53°C for 45 seconds and 72 °C for 45 seconds (35 cycles). The PCR mixture (25 µl) included 1X PCR Buffer (2.5µl), 2 mM dNTPs (1µL), 25 mM MgCl2 (1.2µl), primers (2µl), 0.2U Taq Polymerase (Fermantas) and 5µl DNA. The PCR products were electrophoresed and visualized. The product size is 1204 bp for C626G. For detection of C626G polymorphism, overnight incubation of the product was carried out at 37 °C with 5U of DraIII. Digested products were CC (1204), GG (1005+199), GC (1204+1005+199). Primers used for study were Forward primer: 5′’GGGGACAGGCAGATGGAC’3′ and Reverse primer: 5′’ATCCTCTGGGAACTCTGTGG ’3′ that appeared at 1204 bp after amplification.

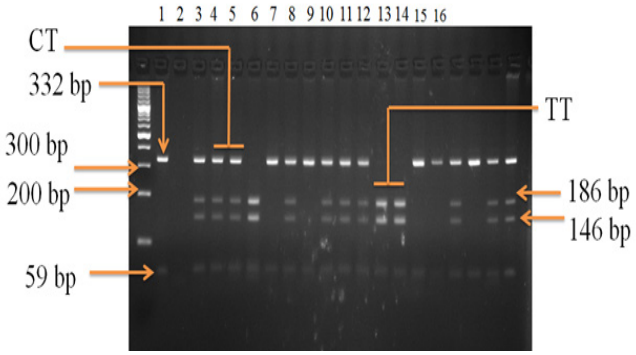

Figure 1. PCR-RFLP gel picture of TRAIL 1595 C/T genotype. Lane 1 showing CC genotype. Lane 4,5 showing CT. Lane 13, 14 showing homozygous TT. CC genotype (Homozygous) was identifiable by the presence of 59 and 332bp. TT genotype was identifiable by the presence of 186, 146 and 59 bp fragments.

PCR-ARMS Method for DR4 A1322G Polymorphism

We used the procedure earlier reported by Taştemir-Korkmaz et al (6) using slight modifications. A1322G polymorphism was studied using PCR-RFLP. Conditions of the PCR were optimized at 95 °C for 5 min, followed by 95 °C for 45 seconds, 53°C for 45 seconds and 72 °C for 45 seconds (35 cycles). The PCR mixture (25 µl) included 1X PCR Buffer (2.5µl), 25 mM MgCl2 (1.2µl), 2 mM dNTPs (1µL), primers (2µl), 5µl DNA, and 0.2U Taq Polymerase (Fermantas). We used 2 separate tubes and forward primer was included in both of the tubes. However, we separately added reverse primers (ending with T and C). Products had 254 bp. We used internal control [CYP19 (Cytochrome P450, subfamily XIX) gene previously reported by Taştemir-Korkmaz et al (6).

Results

TRAIL 1595 C>T genotype

There was no statistically significant role of homozygous CC or TT in HNC. CC was 58% in patients and 49% in controls. CT was 30% in patients and 43% in controls (table 2). TT was 12% in patients and 8% in controls (table 2). PCR-RFLP gel picture of TRAIL 1595 C/T genotype (Figure 1).

Allele frequency for C was noted to be 0.73 (patients) and 0.705 (controls), *p*-value (1). For T, 0.025 (patients) and 0.001(controls), *p*-value (0.88) (table 3).

The genotyping for DR4 gene 626 C>G polymorphism was done for 100 HNC patients and 100 age and sex matched healthy controls. All the genotypes for the polymorphism were in Hardy-Weinberg Equilibrium. Figure 2 showed restriction pattern of DR4626 C>G

Table 2. TRAIL gene 1595 C>T genotypes percentage in HNC patients.

Group	TRAIL 1595 C>T genotype percentage		
	CC	CT	TT
Patient (n=100)	58	30	12
Control (n=100)	49	43	8
***p*-value**	0.384	0.128	0.37

Table 3. Allele frequency of TRAIL gene.

Group	Allele frequency	
	C	T
Patient (n=100)	0.73	0.025
Control (n=100)	0.705	0.001
***p*-value**	1	0.88

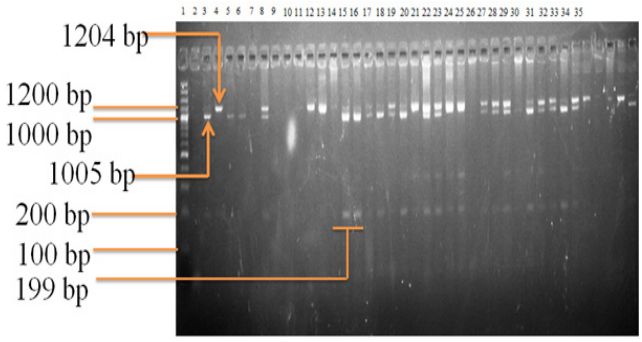

Figure 2. DR4 C626G genotype. Lane 1 is 100bp ladder. Lane 3,5,6 contained GG as evidenced by digested products for GG(1005+199). Lane 4 contained CC (1204) and Lane 8,15,16,17,18,19 contained GC (1204+1005+199).

genotype.

CC was 10% in patients and 2% in controls. GC was 63% in patients and 40% in controls. GG was 27% in patients and 58% in controls (table 4). Interestingly, in DR4 genotyping, CC was predisposing factor and GG acted as a protective factor (table 4).

Allele frequency for C was noted to be 0.41 (patients) and 0.22 (controls), p-value (0.81). For G, 0.585 (patients) and 0.78 (controls), p-value (0.867) (table 5).

DR4 A1322G polymorphism

PCR-ARMS Method for DR4 A1322G Polymorphism

Data obtained from A1322G polymorphism, indicated that TT was 23% in patients and 36% in controls with a *p*-value 0.09 (table 6). CT was statistically significant in patients (45%) and controls (28%), *p*-value 0.04. CC was also non-significant in patients (32%) and controls (36%), *p*-value 0.62 (table 6). C allele was 0.45% in patients and 0.5% in controls. T allele was 0.54% in patients and 0.5% in controls.

Discussion

TRAIL mediated signaling is a deeply studied molecular mechanism and emerging evidence has started to shed light on defective TRAIL induced apoptotic signaling in cancer. Downregulation of death receptors, defective extrinsic and intrinsic pathways pose challenges in standardization of therapy.

TRAIL G1525A and C1595T polymorphisms have earlier been testified to be significantly interconnected with susceptibility of gastric cancer in Chinese Han population (7). However, we were not able to find any significant association between TRAIL polymorphism and HNC susceptibility in our cancer patients (data shown in table 2 and 3).

Laryngeal squamous cell carcinoma (LSCC) is also reported to be associated with defective apoptotic pathway. In a recently published research addressing the relationship of DR4 and cancer progression, increased frequency of the DR4 C626G CC genotype in (LSCC) patients than in controls was noted (8). Contrarily, no association was noted between DR4 gene polymorphism and lung cancer in Turkish population (6). Genetic polymorphism in DR4 gene has previously noted to be associated with bladder cancer in North Indians (9). In our study, for DR4626 C>G genotype, interestingly, CC was predisposing factor and GG acted as a protective factor. Allele frequency for C was noted to be 0.41 (patients) and 0.22 (controls), p-value (0.81). For G, 0.585 (patients) and 0.78 (controls), p-value (0.867).

For the A1322G polymorphism, we did not find any

Table 4. DR4 gene 626 C>G genotypes percentage in HNC patients.

Group	DR4 626 C>G genotype percentage		
	CC	GC	GG
Patient (n=100)	10	63	27
Control (n=100)	2	40	58
***p*-value**	0.02	0.02	0.0007

Table 5. Allele frequency of DR4 gene.

Group	Allele frequency	
	C	G
Patient (n=100)	0.41	0.585
Control (n=100)	0.22	0.78
***p*-value**	0.81	0.867

Table 6. Analysis of exon10 of DR4 with ARMS technique.

Group	DR4 A1322G genotypes percentage			Allele frequency	
	TT	CT	CC	C	T
Patient (n=100)	23	45	32	0.45	0.54
Control(n=100)	36	28	36	0.5	0.5
***p*-value**	0.09	0.04	0.62	0.95	0.96

significant correlation between HNC and homozygous genotype (data shown in table 6). However, heterozygous CT was statistically significant as evidenced by increased frequency in patients (45%) and reduced frequency in controls (28%), p-value 0.04.

Polymorphisms associated with apoptosis related genes are insufficiently studied in cancer patients of our population. Future studies must converge on somatic mutations of DR4 and DR5, association of stages of cancer with DR4 mutation/polymorphisms.

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