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Single nucleotide polymorphisms in ATM, TNF-α and IL6 genes and risk of radiotoxicity in breast cancer patients

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Highlights

- Radiotherapy aims at tumor control with the least damage to healthy tissue.
- The toxicity of the treatment may be acute and / or chronic.
- Radiosensitivity is a multifactorial and polygenic trait.
- SNPs in ATM, IL6 and TNF are key in clinical radiosensitivity.

ABSTRACT

Although oncological therapies have improved in the last decades, breast cancer (BC) remains a serious health problem worldwide. Radiotherapy (RT) is one of the most frequently used treatments for cancer aimed at eliminating tumor cells. However, it can also alter the surrounding normal tissue, especially the skin, and patient reactions may vary as a result of extrinsic and intrinsic factors. We evaluated the association of gene polymorphisms ATM Asp1853Asn, IL-6 G-174C and TNF-α G-308A involved in central phenotype pathways and development of individual radiosensitivity in BC patients with an exacerbated response to RT. Although univariate analysis results did not show a significant association with this trait, the interaction analysis between polymorphisms showed an increased risk of patients presenting wild-type TNF-α G-308A genotype and mutant IL-6 G-174C genotype, and heterozygous TNF-α G-308A genotype and heterozygous IL-6G-174C genotype. On the other hand, our results showed that breast size and patient age influenced the determination of RT-associated effects. Considering that the trait is multifactorial, other significant elements for the determination of individual radiosensitivity should be considered, together with the establishment of specific polymorphic variants.

Keywords: Radiotherapy; Breast cancer; Radiosensitivity; Polymorphisms

1. Introduction

Female breast cancer (BC) mortality and morbidity is very high worldwide and is expected to rise in the next decades, with approximately 115,881 new cases and 32,014 deaths in 2012 in South America [1,2]. In this context, treatment optimization is of great importance. Although different therapeutic tools such as surgery, chemotherapy and hormone therapy are available, radiotherapy (RT) has gained relevance in cancer treatment due to technological and informatics advances, thus becoming a mainstay for most patients diagnosed with this disease [3,4]. RT is always used after conservative surgery and occasionally after mastectomy to eliminate possible residual tumor cells. Although RT is aimed at eliminating tumor cells, healthy cells in the field of irradiation can also be injured, and while normal tissues are able to repair, repeated exposure to radiation generates an imbalance between damage and tissue recovery [5].

Normal tissue injury can be both acute and late. The former develops during treatment and is usually reversible, whereas the latter can occur from six months after RT and even several years later, and can be permanent [6,7]. Skin injuries may include mild to severe dermatitis, ulceration, fibrosis, telangiectasias and even radio-induced cancers [8]. Despite the use of similar protocols, radiotoxicity effects vary among patients, which cannot be explained only by extrinsic factors but could also be attributed to underlying genetic differences [9]. The main causal elements of individual radiosensitivity are related to physics (radiation dose, dose rate, dosimetry, dose inhomogeneity and volume of treatment), to the interaction with other therapies such as surgery, chemotherapy and hormone therapy, and to the patient condition (age, haemoglobin levels, smoking, co- morbidities such as diabetes and vascular diseases). On the other hand, intrinsic factors are concerned with individual radiosensitivity as a multifactorial and polygenic trait, involving DNA repair pathway genes, oxidative stress, endothelial damage and inflammatory response, among others [10,11]. In this context, considering the importance of radiogenomics as a potential tool in the individual radiosensitivity determination and the efforts that are being developed in different laboratories worldwide, for the validation and/or determination of the

different polymorphisms that are part of this feature is interesting the study of particular populations never before analyzed, such as Argentina.

Considering the central effects of ionizing radiation, ATM protein product plays a key role in the detection and repair of radio-induced double-strand breaks (DSBs) mediating this response by phosphorylating numerous substrates involved in specific signalling pathways [12]. Epidemiological evidence suggests that ATM functional genetic variants may have an impact on the risk of presenting RT-induced side effects [13]. However, the holistic approach of modern radiobiology complements this *target theory*, arguing that the pathogenesis of normal tissue is centralized in the inability to repair and recover cells because the microenvironment perpetuates chronic deterioration. This environment is established through feedback of free radicals, cytokine and chemokine cascades that provide a progressive inflammatory response over time. Among these elements, interleukin 1/6 (IL1, IL-6) and tumour necrosis factor alpha (TNF-α) are the most frequently studied [14]. In this way, ionizing radiation is able to stimulate the immune system and promote the bystander effect, thus amplifying the effect of RT [15]. Accordingly, apical kinases such as ATM and pro-inflammatory cytokines play a leading role in the establishment of radiotoxicity as a phenotype. Therefore, the determination of single nucleotide polymorphisms (SNPs) in these genes could be particularly interesting for their relative contribution to radiogenomics, which attempts to establish the influence of genetic variation on the response to radiation.

The aim of this study was to evaluate the association between risk of severe skin reactions in BC patients and ATM G1853A, TNF-α G-308A and IL6 G-174C polymorphisms. These elements would contribute to determine the genetic profile of response to RT as a tool for preventing radiotoxicity and optimizing treatment in the field of precision medicine [16].

2. Materials and methods

2.1. Subjects

Following surgery, BC patients who underwent RT (n=125) were recruited during 2012-2016 at the Oncology Integrated Center (*CIO-La Plata, Terapia Radiante SA*), the main RT center in La Plata, one of the most demographically important areas of Argentina. Of these, 14 individuals who developed severe radiodermatitis (grades 3 and 4) according to the RTOG (Radiation Therapy Oncology Group) score were selected and 14 other individuals were used as controls (grade 0). Grade 3 is characterized by confluent moist desquamation and severe edema, while grade 4 presents with haemorrhages, ulceration and necrosis [17]. The occurrence and severity of these reactions were determined during and after treatment, taking into account the highest degree of toxicity achieved.

Before treatment, 5 mL of peripheral blood was extracted in sterile tubes containing EDTA. In some cases, oral swabs were also collected. Patient data (age, ethnicity, alcohol consumption, smoking, co-morbidities, body mass index and breast size) were recorded through an interview.

All patients were treated with conventional 3D external beam therapy (total dose administered, 50-50.4 Gy, delivered for 5 weeks in daily fractions of 1.8-2 Gy).

Each patient was informed about the study protocol, both verbally and in writing. Only patients who gave their informed consent were included in the study, in accordance with the Declaration of Helsinki.

2.2. DNA isolation and polymorphism genotyping

ATM G1853A (rs1801516), TNF-α G-308A (rs1800629) and IL6 G-174C (rs1800795) polymorphisms were analyzed by pyrosequencing. Genomic DNA was isolated from peripheral blood and oral mucosa samples, according to a protocol previously reported [11]. DNA quality was evaluated by spectrophotometry (Nanovue, GE Heathcare, Buckinghamshire, UK). The target sequence containing the polymorphic site was amplified by polymerase chain reaction (PCR) in which one of the primers was biotinylated. PCR was performed in a total volume of 50 µL containing ~100 ng genomic DNA, 0.2 mM dNTP mixture, 1-2 mM Cl_2Mg, 1.25 U Taq

DNA polymerase (Genbiotech, Buenos Aires, Argentina) and 0.5 pmol/μL primer pairs (Table 1). Reaction conditions were as follows: initial denaturation at 94 °C for 3 min and 35 cycles at 94 °C for 30 sec, annealing at 55-57 °C for 30 sec, elongation at 72 °C for 30 sec and final elongation at 72 °C for 2 min. After amplification, 20 μL biotinylated amplicons were taken to generate a single-strand template by removing the non-biotinylated strand on streptavidin-coated beads (matrix). The DNA template bound to the affinity matrix was separated by denaturation in NaOH and used for the following synthesis of a short-strand DNA (10-15 bases) adjacent to the SNP site. The specific sequence primers are also reported in Table 1. Polymorphisms were analyzed using pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden) according to a previously published method [18].

2.3. Statistical analysis

Allele compliance with Hardy-Weinberg equilibrium and differences in clinical factors between cases and controls were assessed using chi-square test (p-value <0.05). Odds ratios (OR) and 95% confidence intervals (CI) were calculated considering the genotypic dominant model of inheritance. Statistical analyses were performed using IBM SPSS v.19 (IBM Co., Armonk, NY). The interactions of the genes analyzed with the development of skin radio-induced toxicity were detected by the multifactor dimensionality reduction (MDR) software (version 3.0.2) [19].

3. Results

The sample analyzed in this study corresponds to 11.3% of the total patients recruited (patients with severe skin effects grade 3 and 4; mean age, 58 years).

Univariate analysis results showed that patients with severe radiodermatitis differed from controls regarding advanced age in relation to the median value (>59 years) (OR= 6.6, p= 0.02) and breast size (OR=10.4; p=0.04), since patients with large and medium-sized breasts had a

higher risk of developing severe skin reactions (Figure 1). In addition, the analysis performed taking into account other factors such as hypertension, diabetes, alcohol consumption, smoking and body mass index showed no significant differences between cases and controls.

On the other hand, sample genotyping was successfully performed using pyrosequencing, a simple technique for accurate and consistent analysis of large numbers of short- to medium-length DNA sequences. Pyrograms of each genotype corresponding to the polymorphisms studied are shown in Figure 2. The genotype distribution of patients included in the analysis is shown in Table 2. All of them were in Hardy-Weinberg equilibrium ($p > 0.05$), and the homozygous minor alleles of ATM and TNF-α were absent in the population. The frequencies of variant alleles in the whole set of BC patients were 0.16 (ATM Asp1853Asn), 0.04 (TNF-α G-308A) and 0.45 (IL6 G-174C). After the statistical analysis of association of ATM Asp1853Asn, TNF-α G-308A and IL6 G-174C polymorphisms with development of grade 3 and 4 radiodermatitis, no significant differences were observed in OR increase/decrease ($p < 0.05$; Table 2). Interaction analysis results of the association between severe radiotoxicity and TNF-α G-308A and IL6 G-174C polymorphisms were significant, presenting a CVC of 10/10 and a balanced accuracy of 0.6382. Thus, we could identify two risk groups: patients with heterozygous TNF-α G-308A genotype and heterozygous IL6 G-174C genotype, and wild TNF-α- G-308A genotype and mutant IL6 G-174C genotype (Figure 3).

4. Discussion and conclusions

Initially, individual radiosensitivity was studied through cytogenetic and cellular tests such as the comet assay, G2 and cell survival and their combination [20,21,22,23]. However, none of these methods has been validated in a clinical setting. Currently, interest is focused on the investigation of genetic variation, due to advances in genetics, molecular biology and bioinformatics. It is proposed that radiosensitivity studies should be based on the genotype of the

patients, instead of phenotypic aspects, such as cellular radiosensitivity, thus giving rise to radiogenomics.

This study is part of a major project in which polymorphisms are studied in genes involved in various radio-induced routes, such as oxidative stress, DNA repair, cell cycle control and other pro-inflammatory cytokines.

Considering that ATM protein products are central in DNA damage response (DDR), we assessed their relative involvement and verified their role as risk alleles in establishing the toxicity to normal tissue triggered by RT. ATM serine/threonine kinase itself plays a crucial role as a sensor in the activation of cell cycle checkpoints in response to RT-induced DNA DSBs [24]. Genetic variants can lead to structural and functional protein changes, with the consequent defect in the ability of cells to sense and repair DNA damage [25]. In this way, ATM Asp1853Asn (rs1801516) should be evaluated as a common missense variant located in exon 39, and the G to A change leads to the substitution of asparagine for aspartic acid at amino acid position 1853. Although our results are in agreement with the meta-analysis of Su *et al*. [13], who showed that ATM Asp1853Asn polymorphism does not contribute to the development of RT-induced side-effects, this variant was neither present in patients with radiotoxicity nor in asymptomatic patients of our study population. Results with respect to this SNP are controversial since other studies suggest that the 1853Asn allele contributes to the risk of developing severe effects in normal tissue in BC and prostate patients undergoing RT [26].

On the other hand, we also evaluated essential elements of the inflammatory response in the establishment of individual radiosensitivity. Further from causing a direct damage to cells, radiation into the skin also induces inflammation, releasing cytokines such as IL-6, IL1, IL-8 and TNF-α that are able to stimulate the immune system and promote local bystander effects [27].

Interleukin 6 (IL-6) has a regulatory role in cell proliferation, differentiation and the balance between pro-inflammatory and anti-inflammatory pathways. IL-6 promoter contains several SNPs, of which -174G> C is the most widely studied for its influence on various cancers [27].

On the other hand, Siva *et al*. [28] mention that the activation of macrophages by radiation induces the production of IL-6 that mediates acute and late tissue response. However, in the present study, the univariate analysis did not corroborate their participation in these effects in normal tissue.

TNF-α G-308A (rs1800629) polymorphism is in the promoter lying 308bp upstream of the transcription start site. Whereas Talbot *et al.* [29] found association between TNF-α rs1800629 and breast radiotoxicity in a study of 340 individuals, our results showed no association between radiosensitivity and the polymorphism analyzed. This could be explained by the fact that the G and A alleles did not significantly alter TNF-α expression, as reported by Mekinian *et al*. [30].

Regarding the interaction between IL-6 G-174C and TNF-α G-308A polymorphisms, the effect of IL-6 may be more important when interacting with TNF-α, as reported by Schindler *et al*. [31]. These authors mention that the expression of TNF-α is influenced by IL-6. In this way, the wild variants of IL-6 would allow the suppression of TNF-α transcription and therefore a lesser contribution to radiotoxicity, whereas the polymorphism detected in IL-6 would not exert this effect and activate the transcription of necrosis factor, exacerbating the risk of presenting acute effects.

The analyzed genes play a hierarchical role in different routes of response to radiotherapy; however, their impact on the establishment of RT-induced toxicity has not been proven. These data should be confirmed with a larger number of samples. The complexity of the underlying genetic model proposes that increased radiosensitivity could be conferred from a single rare mutation with a large effect to a combination of multiple common variants jointly [8,32].

Considering other factors influencing this phenotype, our results suggest that older patients and larger breasts have an increased risk of radiodermitis. There is conflicting evidence of an effect of age on the prevalence and severity of radiotoxicity [33,34]. In this study, we found that patients older than 59 years were at higher risk for acute effects. Advanced age in adults is generally associated with a decreased normal tissue tolerance. The progressive reduction of the

functional reserve due to depletion of tissue stem cells could increase normal tissue damage and the risk of complications [35]. On the other hand, larger breast volumes require larger doses to be applied to the skin to achieve the desired dose in tissue and deeper structures [34]. Large areas of skin and folds of the breast are exposed to radiation by increasing breast volume. In agreement with Back *et al*. [36], the present results corroborate that patients with medium and large breasts show a more severe skin reaction. Besides, although factors such as BMI, tobacco, alcohol, concomitant diseases (diabetes, hypertension) have been analyzed, these were not significant for severe acute skin reactions. This could be due to the small number of patients presenting these characteristics and to the sample size. In addition, there is no solid evidence that these elements, except BMI, represent risk factors for acute clinical radiosensitivity.

According to the above mentioned and the multifactorial nature of the trait, different elements such as breast size and age of the patients should be considered together with the polygenic component. Although radiogenomics has led to a better understanding of the pathways involved in the determination of individual radiosensitivity for a few years, the involvement of the tissue microenvironment forces the evaluation of this response to a broader and unclarified level of complexity.

Conflict of interest

The authors declare no conflict of interest.

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Table 1

Sequences of the forward, reverse and internal primers for pyrosequencing.

Gene	*SNP*	Primer	Sequence (5´- 3´)	Fragment Size
ATM	G1853A	F- PCR ⟶ R- PCR ⟵● *Sequencing* ⟶	F- GTCAGACTGTACTTCCATACTT R- TGAAAAATCCCTGAACAT S- TTCATGATATTTTACTCCAA	99 bp
TNF-α	G -308A	F- PCR ●⟶ R- PCR ⟵ *Sequencing* ⟵	F- GGAGGCAATAGGTTTTGA R- GCCACTGACTGATTTGTG S- GGCTGAACCCCGTCC	74 bp
IL-6	G-174C	F- PCR ⟶ R- PCR ⟵● *Sequencing* ⟶	F- TTCCCCCTAGTTGTGTCT R- GGAAAATCCCACATTTGA S- CCCTAGTTGTGTCTTGC	132 bp

⟵● biotinylated primer

Table 2

Association between ATM G1853A, TNF-α G-308A and IL-6 G-174C SNPs and manifestation of radiodermatitis throughout the treatment.

Gene	Genotype	n (%)	Radiodermatitis		OR	95% CI	p
			Grade 0	**Grade 3-4**			
TNF-α G-308A	CC	26 (93)	14	12	1		
	CT	2 (7)	0	2	**5.83**	0.5-133.80	0.22
	TT	0	0	0			
	C	27 (96)	14	13	1		
	T	1 (4)	0	1	3.23	0.12-85.59	0.46
IL-6 G-174C	GG	6 (21)	2	4	1		
	GC	19 (68)	10	9	2.16	0.17-27.07	0.54
	CC	3 (11)	2	1			
	G	15,5 (55)	7	8,5	1		
	C	12,5 (45)	7	5,5	1.54	0.14-2.89	0.57
ATM G1853A	GG	19 (68)	9	10	1		
	GA	9 (32)	5	4	0.72	0.14-3.54	0.68
	AA	0	0	0			
	G	23.5 (84)	11.5	12	1		
	A	4.5 (16)	2.5	2	0.76	0.10-5.82	0.79

Figure 1

Comparison between patients who developed grade 3 and 4 severe radiodermatitis and asymptomatic patients. **A.** In relation to age, it is observed that those patients with a median age (>59 y) are more at risk of developing severe dermitis. **B.** Regarding breast size, it has been observed that patients with medium and large breasts are more at risk of presenting radiodermitis than patients with small breasts.* represents p <0.005.

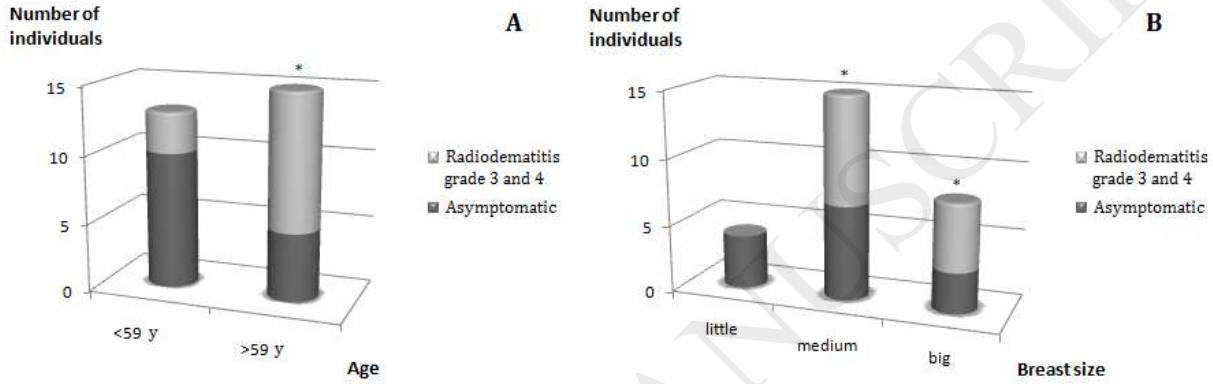

Figure 2

Pyrograms obtained after genotyping ATM G1853A, TNF-α G-308A and IL-6 G-174C polymorphisms. The yellow area shows the peaks that represents the resulting genotypes. The theoretical histogram is shown below each one.

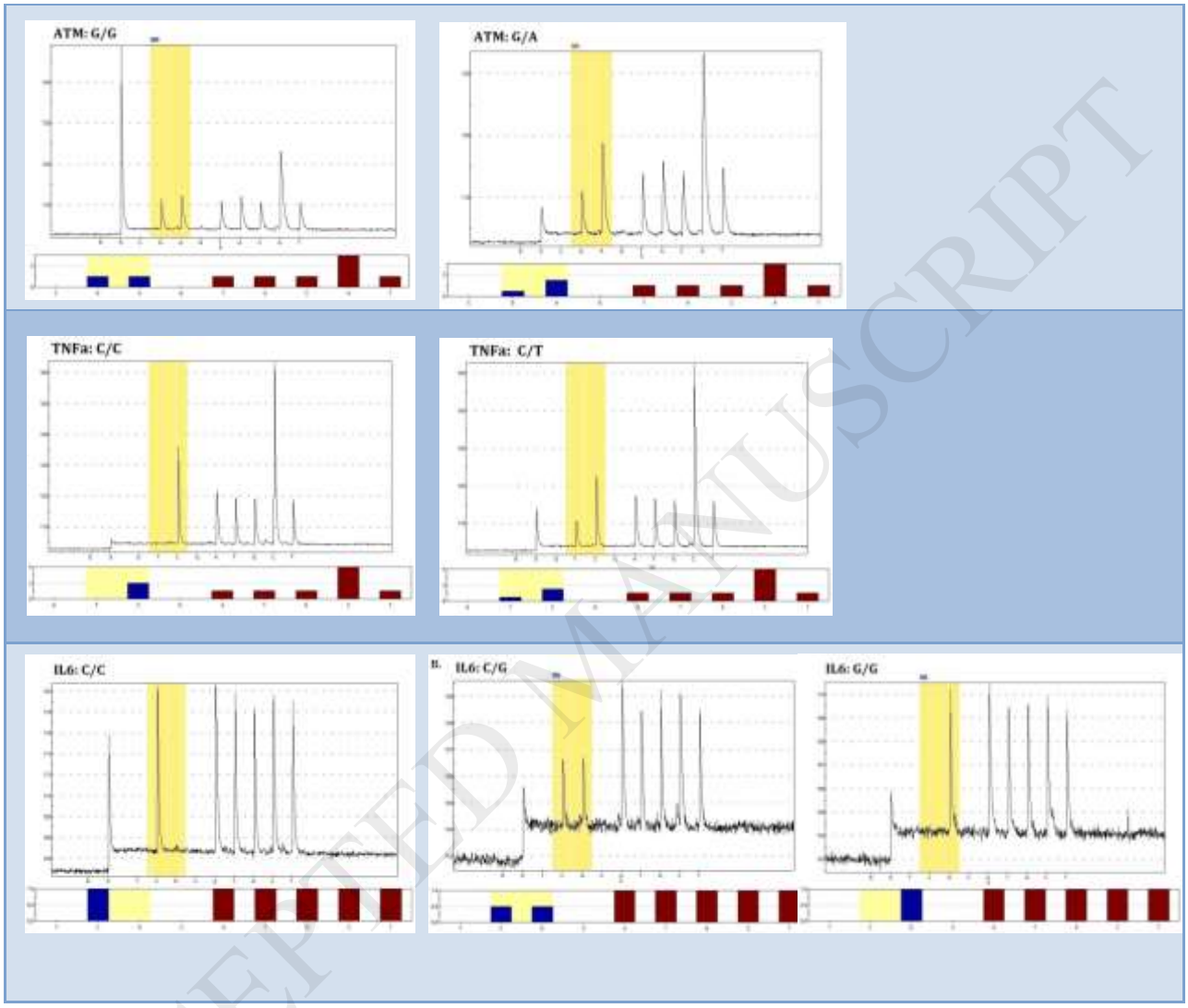

Figure 3

Interaction analyses with MDR. **A.** Interaction model for TNF-α G−308A and IL-6 G−174C polymorphisms when comparing the groups with severe radiotoxicity (left bars) and controls (right bars), which are illustrated for each multilocus genotype combinations. Light gray cells are labelled as low risk and dark gray cells are labelled as high risk. White cells indicate that there are no individuals with combinations of these genotypes. **B.** Entropy decomposition. The effect provided by TNF-α G−308A individually is higher than the effect resulting from the interaction between these two genes in establishing radiotoxicity risk.