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Genotype of inflammatory cytokines in limbal stem cell graft in Italian patients

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Abstract

We tested the hypothesis that the genetic capability to mount an inflammatory response might contribute to the inter-individual variability of limbal stem cell graft (LSCG) outcome. Two functional polymorphisms in the IL-6 and TNF-α promoter regions were genotyped in 35 patients. A new score system (clinical assessment score, CAS) was set up in order to classify patients' clinical profile, and the main parameters relevant for LSCG as well as for the follow-up of the patients. Patients carrying at both loci a genotype associated with a lower production of both cytokines were classified as "low producers" (LP), while all the others were classified as "intermediate or high producers" (HP). LP patients did not show any difference in CAS before and after transplantation while a significant difference was present in HP patients. A similar trend was evident in the 35 months of follow-up. Polymorphisms of IL-6 and TNF-α can be used to identify subgroups of patients with higher risk of unsuccessful outcome.

Keywords: Limbal stem cell allograft; Inflammation; Genetics; IL-6; TNF-α

Limbal transplantation is required to restore the ocular surface of patients with stem cell deficiency [1,2] caused by different pathological conditions: chemical burns [3,4], pemfigo, and Stevens–Johnson disease [5,6].

Although surgical procedures are well established [7,8], the recipient environment hosting donor stem cells plays a primary role in determining the graft's outcome by influencing donor stem cell apoptosis and proliferation capacity [9].

The major component of this microenvironment alteration is proved to be the inflammatory process, pre-existent or caused by surgical procedures or by the allograft itself [10,11]. As in other tissue transplants, a high inflammatory condition can result in a strong impairment of the graft to survive.

Drugs or the use of an amniotic membrane's matrix has been shown to suppress the production of inflammatory cytokines, thus significantly improving the outcome of limbal transplantation and patient recovery [12–15]. Within this scenario a great variability in individual conditions and responses to limbal allograft is reported.

The production of inflammatory cytokines is well established to be dependent on the presence of different alleles in regulatory regions of the respective genes [16,17], and in particular interleukin-6 (IL-6) −174 and tumor necrosis factor-α (TNF-α) −308 polymorphisms

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are extremely relevant in such pathological conditions with an inflammatory background [18–24].

The present work investigates the possible association of the genotype of IL-6 and TNF-α with limbal transplant's outcome.

Materials and methods

Thirty-five patients with pathological conditions for which a limbal transplant was recommended, i.e., chemical burns, pemfigo, and Stevens–Johnson disease, were asked to participate in the study and to give their informed consent. The patients were evaluated immediately before and after the surgery (keratolimbal allograft) at regular intervals or when needed, until a maximum of 35 months after the transplant. To evaluate the possible role of genetic markers in the outcome of keratolimbal allograft, a new score system was set up in order to classify patients' clinical profile, taking into account the main parameters relevant for the choice of a limbal transplant as well as for the follow-up of the patients.

A score ranging from 0 (the best) to 100 (the worst) was calculated, as a result of four main areas contributing to the clinical evaluation. Such a score allowed us to take into account the post-transplant condition, as it included the rejection response and the recovery data. The final value was called clinical assessment score (CAS) as shown in Table 1.

To investigate the association of IL-6 and TNF-α polymorphisms with the CAS, as well as its modifications after the transplant, genomic DNA was purified from peripheral blood lymphocytes of each patient and genotyped with RFLP methods. In particular, IL-6 genotype was determined according to Olomolaiye et al. [25] and TNF-α according to Wilson et al. [26] with the following modification of primer pairs: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and 5′-CAGCGGAAAA CTTCCTTGGT-3′. CAS value distributions were evaluated with a normal Q–Q plot and tested with the Shapiro–Wilk normality test. The results of such evaluation suggested to proceed with nonparametric tests. Accordingly, the scores of each patient were analyzed with the Wilcoxon signed rank test and the follow-up was described with a Kaplan–Meier function, using the Yates' correction to the log-rank test.

Results

According to CAS, the available clinical data were clustered in four main areas considered of critical importance in limbal transplant, according to the literature and to our experience. The four areas were the following: (i) general ocular evaluation data, referring to the presence of keratinization or metaplasia conditions; (ii)

Table 1
Clinical assessment score

Clinical feature	Diagnosis	Score	Area weight
Ocular evaluation data			0–25
Keratinization or metaplasia	Nonevident metaplasia	0	
	Low squamous metaplasia	3.5	
	Moderate squamous metaplasia	7	
	Corneal advanced metaplasia and keratinization	10.5	
Lacrimal film	Optimal	0	
	Moderate ipo-lacrimia	3.5	
	Evident ipo-lacrimia	7	
	α-Lacrimia	10.5	
Ocular annexes	Normal	0	
	Pathologic	4	
Inflammatory condition data			0–25
Melboimitis	Absent	0	
	Low melboimitis	4.1	
	Moderate melboimitis	8.2	
	High melboimitis	12.4	
Conjunctival inflammation	Absent	0	
	Low inflammation	4.2	
	Moderate inflammation	8.4	
	Severe inflammation	12.6	
Rejection data			0–25
Rejection[a]	Absent	0	
	Late rejection	12.5	
	Acute rejection	25	
Recovery data			0–25
Ocular surface stability[a]	Stable	0	
	Unstable	25	
Total score			0–100

Given a maximum score of 100 (worst condition), the score was subdivided into the four majors area of interest and further subdivided in clinical factors contributing to the evaluation. The higher is the score, the worse is the clinical condition.

[a] To be evaluated after the surgical procedure.

inflammatory conditions, such as melboimitis or conjunctival inflammation; (iii) the presence of rejection after surgery; and (iv) the ocular surface relative stability.

Within a maximum score of 100 (the worst), 25 points were attributed to each of the four main areas, and within each area the 25 points were assigned to the contributing factors as reported in Table 1.

This approach allowed us to obtain a new score (CAS) which was more suitable than previously reported scores [27,28] for the purpose of the present investigation and specifically tailored to evaluate the role of inflammatory response and inflammatory genotypes in the final outcome of limbal stem cell graft.

In the 35 patients analyzed, a nonnormal distribution was observed according to the Q–Q plot, both as far as the CAS before transplant (Skewness = 0718; Kurtosis = −0598) and after transplant (Skewness = 0694; Kurtosis = −1080) was concerned.

Moreover, the value distribution after surgery was quasi- bi-modal, suggesting the presence of two groups responding differently to the clinical procedure.

The simultaneous assessment of the genotype of IL-6 and TNF-α genes at position −174 and −308, respectively, allowed us to evaluate the possible role of the inflammatory genetic profile.

Those patients carrying at both loci a genotype associated with a lower production of the cytokines, thus characterized by a lower inflammatory response, were classified as "low producers." Conversely, those patients carrying at both loci a genotype associated with a higher production of the cytokines or having at least one genotype associated with a high production of one of the two cytokines were referred to as "intermediate or high producers."

According to this classification, "low producers" display a similar CAS before and after the treatment, while "intermediate or high producers" show a clinical score significantly increased after the surgery procedure ($p = 0.009$; Fig. 1).

Specific examples of such patients are illustrated in Fig. 2. In particular, Fig. 2A shows the ocular surface of a high producer patient before (A1) and after (A2) the transplant, while Fig. 2B displays the ocular surface of a low producer patient before (B1) and after (B2) the transplant. While the patient illustrated in Fig. 2B shows a definite recovery of the surface, patient illustrated in Fig. 2A shows a persistent inflammatory response, associated with extensive vascularization considered a sign of unsuccessful outcome [28,29].

These results, while indicating a genetic contribution to the clinical outcome of the limbal stem cell transplant, left open the question of the importance of the genotype of the pro-inflammatory cytokines investigated for the long-term success of the transplant. The follow up of the patients for a maximum of 35 months clearly indicates that even this parameter is affected by IL-6 genotype, as reported in Fig. 3. In order to obtain the survival curves reported in Fig. 3, we analyzed the distribution of the delta-score, i.e., the difference between pre- and post-surgery CAS. This distribution resulted in a bi-modal shape, and the cutting point between the two internal distributions was located in correspondence of a difference between the scores of 20 points. Accordingly a delta value of 20 was assumed as a threshold for the analysis, and the exit event of the survival function was fixed according to the time (in months) when this threshold was crossed during the follow-up period (Fig. 3). As reported in Fig. 3, it is evident that patients having an inflammatory genotype (*C noncarriers*, as referred to the IL-6 −174 alleles) have a tendency to a worst outcome after limbal transplantation in comparison with *C carriers*. This difference, although not statistically significant, suggest a possible role of inflammatory polymorphisms in the long term clinical evolution of limbal transplant (log-rank = 1.59 and $p = 0.1937$). To this regard the results obtained using the polymorphism at −308 position of TNF-α are less clear and no tendency to a correlation between this polymorphism and the long term outcome emerged (data not shown).

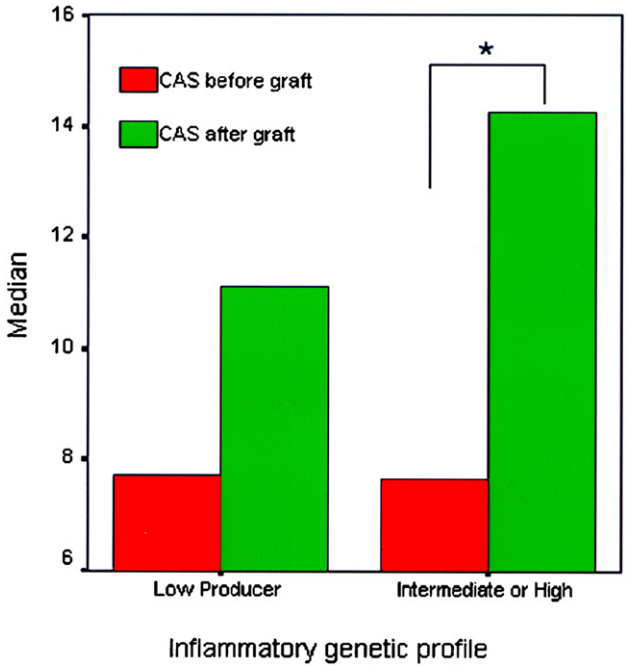

Fig. 1. Difference between CAS median values before and after the limbal stem cell graft in the groups of "low producer" and "intermediate/high producer," according to their inflammatory genetic profile. The statistical analysis showed no differences between CAS level in low producers patients, while a significant difference was found in intermediate/high producers (Wilcoxon signed rank test, $p = 0.009$).

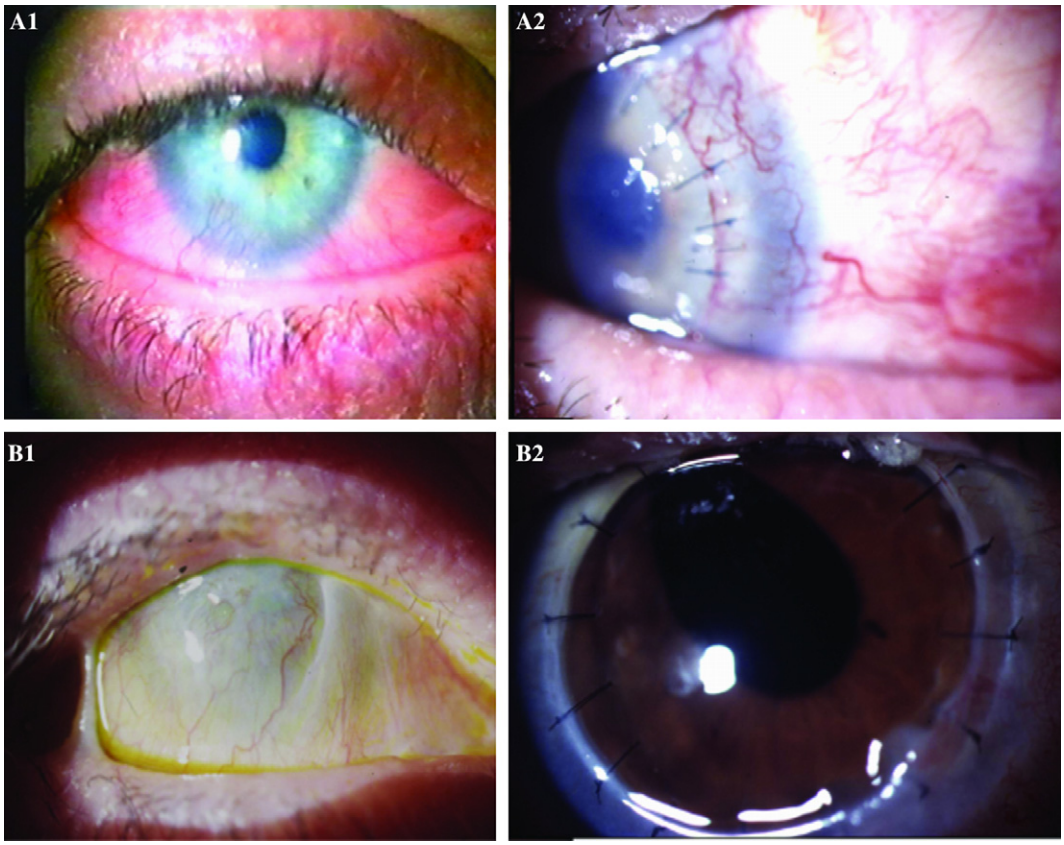

Fig. 2. The ocular surface before and after keratolimbal allograft in a patient with a high producer genetic profile (A1 before, A2 after the transplant) is shown, in comparison with the ocular surface of a patient with a low producer genetic profile (B1 before and B2 after the transplant). In the high producer patient, a persistent inflammatory response is evident (A2), together with an extensive vascularization, a hallmark of unsuccessful outcome.

Discussion

Limbal stem cell allograft is an established procedure to repair damaged corneal tissue resulting from a variety of insults and pathological conditions [1–6]. However, a great individual variability is observed as far as the short- and long-term success of the transplant is concerned. The reasons for such an individual variability are largely unknown. In this paper, we tested the hypothesis that the individual capability to mount an inflammatory response is a candidate to explain at least in part such inter-individual variability [30]. To this aim, two polymorphisms in the promoter region of well-known inflammatory molecules such as IL-6 and TNF-α gene, known to be related to the cytokine production [18–20,31,32], were evaluated.

The major finding of this paper is that a genetic profile which combines the genotypes of IL-6 and TNF-α appears to be correlated with the clinical status of the transplant, thus offering a new marker to be used in clinical practice. Moreover, we show that there is a tendency towards a correlation between the long-term outcome of the limbal transplant after a maximum of 35 months of follow-up and the −174 IL-6 polymorphism. The apparent failure of −308 TNF-α polymorphism to show even a tendency to a correlation with long term outcome of limbal transplant may be related to the possible role of this cytokine in the early phase of the graft response rather than to the final outcome. Such a hypothesis deserves further studies in a larger number of patients. The same consideration applies to the other findings presented in this paper.

Finally we would like to stress that in order to evaluate the possible role of IL-6 and TNF-α genetic markers in the clinical outcome of the limbal transplant a new clinical assessment score was set up. This score we called CAS allows a quantification of the major components (four areas regarding ocular evaluation, inflammatory status, rejection, and surface stability) of clinical status and the inflammatory response of transplanted stem cells and of the ongoing repair process. We hope that this new score can be added to the tools used to clinically evaluate limbal transplants, but we are also aware that improvements are possible and likely needed.

In conclusion, as far as we know, our data show for the first time that genetic markers related to well-known proinflammatory cytokines such as IL-6 and TNF-α can be used to identify subgroups of patients which underwent limbal transplants

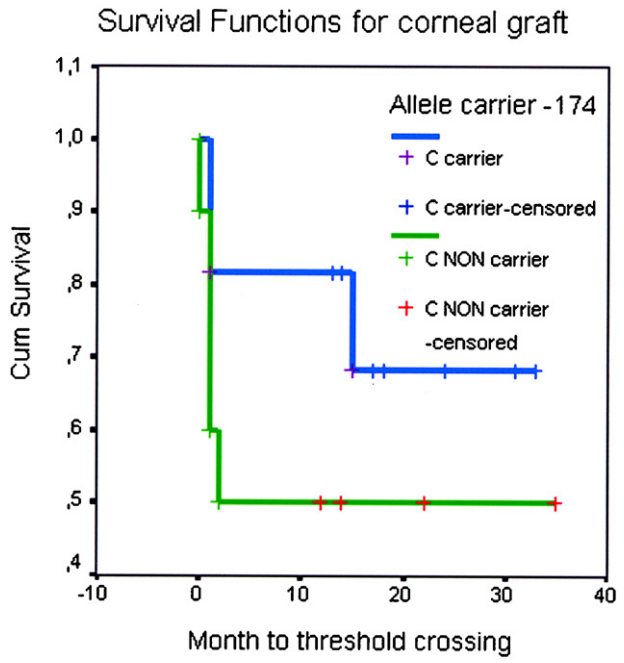

Fig. 3. Survival function of keratolimbal allograft in 35 patients, with a maximum follow-up of 35 months. A delta value of 20 of the CAS before and after the transplant was assumed as a threshold for the analysis, and the exit event of the survival function was fixed according to the time (in months) when this threshold was crossed during the follow-up period. Patients carrying an allele associated with a high production of interleukin 6 at −174 position (*C noncarriers*, green line) display a worst outcome after the surgical procedure, in comparison with *C carriers* (blue line). (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

characterized by a higher risk of unsuccessful outcome. Patients with the genetic risk profile associated with high production of IL-6 and TNF-α could represent a target for a more intense monitoring and anti-inflammatory interventions.

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