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# Inconsistent Expression of HLA-B Antigens on Peripheral Blood Lymphocytes of Stage I Melanoma Patients: an Indicator of Poor Prognosis

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**The survival of stage I melanoma patients was evaluated and compared with the detectable expression of HLA antigens. Of 904 patients who were surgically treated, 219 were HLA typed on peripheral blood lymphocytes. Four consecutive HLA typings were considered necessary. Median follow-up was 8 years. Two main groups of patients were considered: (a) patients with consistent detectable expression of antigens; and (b) patients with inconsistent detectable expression of antigens. Patients with consistent HLA antigens detection had an 8-year survival rate of 87.7% compared with 49.2% of patients with an inconsistent rate ($P\ 10^{-7}$). Multivariate analysis of survival of the 182 HLA-typed patients who survived at least 24 months from surgery showed that two of the criteria had an independent impact on survival: tumour thickness ($P$ 0.02) and HLA typing ($P\ 2 \times 10^{-5}$). Inconsistent detection of HLA antigens on peripheral blood lymphocytes during the first 24 months after surgery is an indicator of poor prognosis in stage I melanoma patients.**

**Key words: HLA typing, cutaneous melanoma, prognosis, stage I melanoma**
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## INTRODUCTION

THE PROGNOSIS of patients with cutaneous melanoma still confined to its site of origin (clinical stage I) is assessed on the histological characteristics of the primary tumour. Breslow's thickness and ulceration have been found to be the most important and reproducible prognostic criteria in a major series of publications [1–5]. Other histological features, such as regression [6–8], inflammatory reaction [9], mitotic activity [10] and lymphocytic infiltration [11], are still matters of discussion. Among host characteristics only sex was found to be significantly associated with survival [1].

In a previous study, we found that patients with melanoma showed a higher frequency of failure to express antigens of the HLA locus-B on peripheral blood lymphocytes before surgery compared with healthy donors [12, 13]. Therefore, we have investigated whether detection of HLA antigens is consistent with time, and verified whether a possible inconsistency is associated with survival of stage I melanoma patients.

## PATIENTS AND METHODS

*Patients*

From 1980 to 1983 and between 1985 and 1987, 904 stage I melanoma patients were treated at the National Cancer Institute

*Table 1. Distribution of major prognostic criteria in 734 stage I melanoma patients*

| | No HLA typing (552 patients) | | HLA typing (182 patients) | | Total (734 patients) | | $P^*$ value |
|---|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | *n* | % | |
| Sex | | | | | | | |
| Male | 231 | 41.8 | 59 | 32.4 | 290 | 39.5 | 0.03 |
| Female | 321 | 58.2 | 123 | 67.6 | 444 | 60.5 | |
| Site of tumour origin | | | | | | | |
| Head and neck | 84 | 15.2 | 14 | 7.7 | 98 | 13.3 | |
| Trunk | 165 | 29.9 | 55 | 30.2 | 220 | 30.0 | 0.02 |
| Limbs | 300 | 54.4 | 113 | 62.1 | 413 | 56.3 | |
| Unknown† | 3 | 0.5 | — | — | 3 | 0.4 | |
| Breslow thickness | | | | | | | |
| ≤2.0 mm | 274 | 49.6 | 89 | 48.9 | 363 | 49.4 | |
| >2.0 mm | 233 | 42.2 | 86 | 47.2 | 319 | 43.5 | 0.52 |
| Unknown† | 45 | 8.2 | 7 | 3.9 | 52 | 7.1 | |
| Levels | | | | | | | |
| I | 11 | 2.0 | 2 | 1.1 | 13 | 1.8 | |
| II | 131 | 23.7 | 39 | 21.4 | 170 | 23.2 | |
| III | 175 | 31.7 | 74 | 40.7 | 249 | 33.9 | 0.40 |
| IV | 166 | 30.1 | 58 | 31.9 | 224 | 30.5 | |
| V | 28 | 5.1 | 7 | 3.8 | 35 | 4.8 | |
| Unknown† | 41 | 7.4 | 2 | 1.1 | 43 | 5.8 | |
| Ulceration | | | | | | | |
| Yes | 139 | 25.2 | 61 | 33.5 | 200 | 27.2 | |
| No | 301 | 54.5 | 101 | 55.5 | 402 | 54.8 | 0.19 |
| Unknown† | 112 | 20.3 | 20 | 11.0 | 132 | 18.0 | |

*Related to the frequency of criteria in HLA-typed and non-HLA-typed groups. †These values were not considered in the analysis.

of Milan, Italy. Of these, 219 gave informed consent to enter into this study.

Surgical treatment consisted of wide excision of the primary melanoma (3–5 cm margin) if Breslow's thickness was greater than 2 mm; patients with a primary melanoma up to 2 mm were randomised to receive either wide or narrow (1 cm margins) excision to meet the criteria of a randomised international clinical trial [14] in which we were participating. No elective node dissections were performed. After surgical treatment, patients were followed up every 3 months for the first 2 years, every 6 months for up to 5 years, and once a year thereafter.

The follow-up checks were based on clinical examination, with special emphasis on the assessment of the status of regional lymph nodes. In asymptomatic patients, chest X-rays and liver echotomography were performed every 6 months for up to 5 years and once a year thereafter.

Only patients surviving for at least 24 months were included in the analysis of the influence of antigens on survival, because at least four consecutive HLA typings were considered necessary to evaluate variations of antigen detection for this reason, 37 (16.9%) of the 219 patients who were HLA typed and 136 (19.7%) of the 688 other patients were excluded from the analysis ($P = 0.39$).

The main characteristics of the 734 evaluable patients are summarised in Table 1. The majority of patients were female, and the extremities were the most frequent site of origin. With respect to histological features, most melanomas were classified as levels III and IV, maximum thickness was not greater than 2 mm in 363 patients and ulceration was present in 200 patients. The median follow-up period was 8 years in the two groups.

*HLA typing*

Peripheral blood lymphocytes of melanoma patients were typed for class I and II HLA antigens by the NIH (National Institutes of Health) method [15]. The reactions were considered positive if at least 40% of the expected cell lysis was obtained. Since, from previous studies [12, 13], we had evidence of higher frequency of locus B blanks, only this locus was investigated at the second typing. HLA typings were performed for all patients within 1 week before surgery, 6 months later, and at 1 and 2 years. Most of the survivors were typed once a year thereafter.

Different sera were used in typing during the study. The antiserum currently being used recognises 47 specificities of locus B (Table 2). With the serum available in 1980, we were unable to detect nine specificities, whose frequency in the Italian population is 1.25%; the serum used in 1981–1982 failed to recognise eight specificities that are present in 0.35% of the Italian population. Sera used in 1983 and 1985–1987 missed antigens present in 1 and 0.12%, respectively (see Table 2 for details).

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*Table 2. HLA-B specificities recognised by sera used in different time periods*

| Specificites recognised by present sera | | Specifities *not* present in sera used in the past | | | |
|---|---|---|---|---|---|
| | | 1980 | 1981–1982 | 1983 | 1985–1987 |
| B5 | B50 | B53 | B53 | B53 | B42 |
| B7 | B51 | B41 | B42 | B47 | B47 |
| B8 | B52 | B42 | B46 | B48 | B48 |
| B12 | B53 | B46 | B47 | B41 | B70 |
| B13 | B54 | B47 | B59 | B42 | B73 |
| B14 | B55 | B59 | B48 | B46 | |
| B15 | B56 | B48 | B73 | B59 | |
| B16 | B57 | B73 | B70 | B73 | |
| B17 | B58 | B70 | | B70 | |
| B18 | B59 | | | B48 | |
| B21 | B60 | | | | |
| B22 | B61 | | | | |
| B27 | B62 | | | | |
| B35 | B63 | | | | |
| B37 | B64 | | | | |
| B38 | B65 | | | | |
| B39 | B67 | | | | |
| B40 | B70 | | | | |
| B41 | B71 | | | | |
| B42 | B72 | | | | |
| B44 | B73 | | | | |
| B45 | | | | | |
| B46 | | | | | |
| B47 | | | | | |
| B48 | | | | | |
| B49 | | | | | |

To evaluate possible bias related to the observer, a quality control was planned every 6 months: the typings were conducted simultaneously by three individuals.

The reactivity of the complement was checked on lymphocytes from 50 blood donors of whom the HLA antigens were known whenever a new stock of complement was made available at the laboratory. To check the activity of the antibodies, lymphocytes from a subject with a known HLA profile were added to the routine typings every week.

During the study, the 182 melanoma patients were tested together with 781 healthy donors and 991 patient with other malignancies.

*Statistical methods*

The end point of this evaluation was the date of death of patients from the date of surgery. Only deaths from the related cancer were considered as events. Contingency tables were calculated using the $\chi^2$ method. Survival rates were calculated by means of the actuarial life table approach, breaking the entire series into many subgroups according to all possible prognostic variables. Statistical analysis of the differences between survival curves was by the log-rank test [16].

The multifactorial analysis was carried out using Cox's regression model [17]. The relative rate of each variable was assessed by the step-down procedure for variable selection at the 5% level of significance.

## RESULTS

The frequency of subjects with undectable expression of HLA locus B antigens determined at the first typing is given in Table 3. From this table it may be seen that: (1) the percentage of individuals with undetectable antigens decreased from 1980 to 1987 among healthy donors, and that locus B "blanks" in these subjects was in the range of the expected value at that date; (2) the frequency of locus B "blanks", with the exception of 1983, was always higher in melanoma patients than in healthy donors and in patients with other malignancies. The $\chi^2$ analysis carried out on the total indicates that the frequency of undetectable expression of antigens on peripheral blood lymphocytes of melanoma patients is significantly higher than in patients with other malignancies ($P = 0.01$) and in healthy donors ($P = 0.0004$).

By evaluating the four HLA typings carried out on the same subject during the first 2 years of follow-up, we identified three groups of patients: (1) subjects on whose lymphocytes two class I locus B antigens were always detectable, which we called "consistent" (102 patients), (2) patients whose lymphocytes irregularly had at least one of the two antigens detectable, which we called "inconsistent" (55 patients), (3) patients whose lymphocytes had only one detectable antigen (always the same) (25 patients).

Neither particular locus B antigens nor antigen clusters were involved in this phenomenon.

We cannot assess if the down expression of antigens is related to a general reduction in detectability of antigens because the level of cell lysis was not recorded in the database when the tests were performed.

The first step of the analysis was to verify if major selection biases existed that might impair the comparative evaluation of survival. Table 1 gives the distribution of the most important prognostic criteria in HLA-typed patients and the others. It may be seen that the observed differences in the distribution of clusters of thickness, levels of invasion and ulceration between the two groups were statistically non-significant. In contrast, sex and the related site of origin were unbalanced in the two groups of patients.

We then verified if this unbalanced distribution had an impact on the survival of the two groups: the 8-year survival rate of the HLA-typed patients was 75.5% as compared with 77.3% in the remaining patients (Fig. 1).

We then compared the survival curves of patients who showed only one locus B antigen and consistent patients. The survival of the two groups of patients was very similar (Fig. 2): for this reason, these 25 patients were considered as "consistent" in this analysis. Figure 3 compares survival of consistent patients with survival of inconsistent patients: it may be observed that the first group of patients has a significantly higher survival than the

*Table 3. Comparison of frequencies of undetectable HLA locus B antigens in stage I melanoma patients, patients with other malignancies and healthy donors*

| Year | Percentage of undetectable antigens | | |
|---|---|---|---|
| | Healthy blood donors | Other tumours | Stage I melanoma |
| 1980 | 14/78 (17.94%) | 23/132 (17.42%) | 9/34 (26.47%) |
| 1981–1982 | 33/250 (13.20%) | 65/429 (15.15%) | 24/92 (26.08%) |
| 1983 | 28/159 (17.61%) | 41/263 (15.58%) | 6/37 (16.21%) |
| 1985–1987 | 26/294 (8.84%) | 27/167 (16.16%) | 4/19 (21.05%) |
| Total | 101/781 (12.93%) | 156/991 (15.74%) | 43/182 (23.62%) |

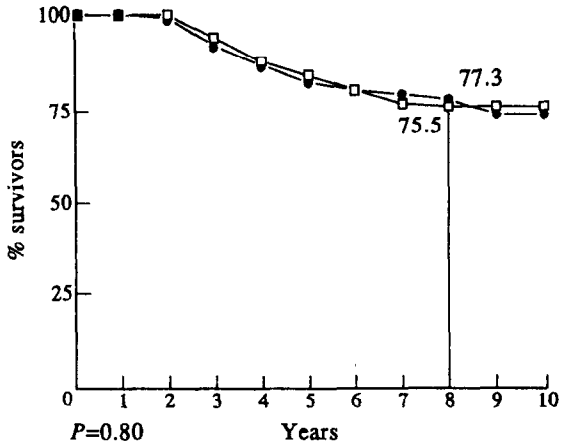


**Fig. 1. Overall survival of 734 stage I melanoma patients. Comparison of survival observed in patients who were HLA typed and those who were not. —□— HLA typing (182 patients); —●— controls (552 patients).**

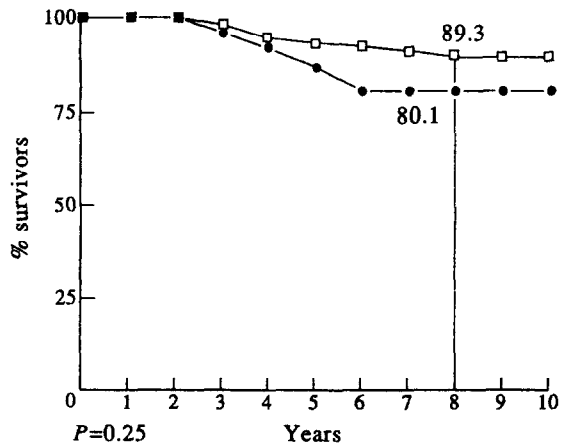


**Fig. 2. Overall survival of 127 stage I melanoma patients. Comparison of survival of patients whose peripheral blood lymphocytes always had two locus B antigens detectable, and of patients with only one antigen detectable. —□— Consistent (102 patients); —●— homozygotes (25 patients).**

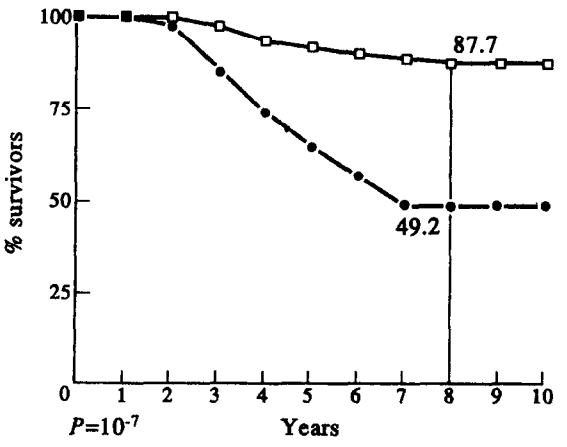


**Fig. 3. Overall survival of 182 stage I melanoma patients. Comparison of consistent patients with inconsistent patients. —□— Consistent and homozygotes (127 patients); —●— inconsistent (55 patients).**

*Table 4. Multivariate analysis of survival of 182 stage I melanoma patients whose peripheral blood lymphocytes were HLA typed*

| Criterion (adjusted by the others) | β | S.E. | RR | $\chi^2$ | *P* | df |
|---|---|---|---|---|---|---|
| Breslow thickness | | | | 5.17 | $2 \times 10^{-2}$ | 1 |
| ≤2.0 mm | −0.405 | 0.18 | 1.00 | | | |
| >2.0 mm | +0.405 | — | 2.24 | | | |
| HLA typing | | | | 18.14 | $2 \times 10^{-5}$ | 1 |
| Inconsistent | +0.710 | 5.7 | 1.00 | | | |
| Consistent | −0.710 | — | 0.24 | | | |

S.E., standard error; RR, relative risk; df, degrees of freedom.

other. The multivariate analysis of survival was calculated considering consistent versus inconsistent patients.

The hypothesis that surgery could remove a hypothetical masking of class I HLA antigens was verified. The percentage of patients that, after surgery, expressed HLA antigen was 17.2 in 157 patients definitively not homozygotes (for this reason the 25 patients who were potentially homozygotes were excluded), while the percentage of patients losing the HLA antigen after surgery was 10.2. This difference was statistically non-significant ($P = 0.10$).

The removal of the tumour did not seem to influence significantly the antigen expression of histocompatibility.

The results of the multifactorial analysis of survival are given in Table 4. It may be observed that two criteria influence survival of the 182 HLA-typed patients: maximum tumour thickness and the outcome of HLA typing.

The unexpected finding in this study was the irrelevance of sex in assessing prognosis of the HLA-typed group. Table 5 shows the survival by sex of HLA-typed patients and the others: it may be observed that, in the last group, females do significantly better than males. For this reason, we carried out a subgroup analysis, taking into consideration tumour thickness, sex and HLA (Table 6). It is noticeable that inconsistent patients have a poorer prognosis in all subgroups of patients.

We have also investigated whether the frequency of undetectable antigens in the 37 patients who were excluded because they died within 2 years of treatment was higher than the frequency observed in HLA-typed patients who survived for at least 2 years. Expression of HLA-B antigens was not detectable in 40.5% of the 37 excluded patients, and in 30.2% of the other 182 patients. This observed difference was statistically non-significant ($P = 0.30$).

## DISCUSSION

The results of this study indicate that patients with stage I melanoma whose peripheral blood lymphocytes have HLA locus

*Table 5. Eight-year survival by sex and HLA typing of stage I melanoma patients*

| | No HLA typing (552 patients) | | HLA typing (182 patients) | |
|---|---|---|---|---|
| Sex | *n* | % 8-year survival | *n* | % 8-year survival |
| Male | 231 | 70.9 | 59 | 78.3 |
| Female | 321 | 82.1 | 123 | 74.4 |

*Table 6. Eight-year survival rates according to sex and tumour thickness in patients with consistent and inconsistent HLA antigen detection*

| | Inconsistent | Consistent | Total |
|---|---|---|---|
| Males | | | |
| ≤2.0 mm | 60.0 (5) | 100 (16) | 92.0 (21) |
| >2.0 mm | 46.7 (15) | 86.5 (23) | 70.2 (38) |
| Females | | | |
| ≤2.0 mm | 64.3 (14) | 89.1 (54) | 83.7 (68) |
| >2.0 mm | 35.7 (21) | 79.9 (27) | 59.5 (48) |
| Total | | | |
| ≤2.0 mm | 66.2 (19) | 91.3 (70) | 85.6 (89) |
| >2.0 mm | 40.4 (36) | 82.9 (50) | 64.1 (86) |

In parentheses, the number of patients.

B antigens detectable by the NIH method at the time of diagnosis, and who consistently demonstrate this characteristic during the first 2 years of follow-up (consistent) have a significantly better prognosis than patients whose lymphocytes have at least one of these antigens irregularly detectable in the same period of time (inconsistent). We considered that at least four consecutive HLA typings were necessary for a good evaluation of the variation of antigen detectability; for this reason, only patients with at least 2 years of follow-up were taken into consideration in this analysis. Patients whose lymphocytes had only one HLA locus-B antigen (always the same) detectable deserve some discussion. These patients may be considered to be homozygotes. We cannot be sure of this assumption because we were unable to perform family studies. However, the fact that one HLA locus B antigen was detectable in at least six consecutive HLA typings, and that the prognosis of these patients is not different from that of consistent patients supports this hypothesis.

Patients' willingness to enter into the study introduced minor biases in the evaluation of prognosis; the two groups considered had the same survival. The different frequency of females in the two series of patients evaluable for survival analysis was due to the exclusion of patients who died or were lost to follow-up during the first 2 years after surgery for primary melanoma. The exclusion rate was similar: 16.9% in HLA-typed patients and 19.7% for the other patients. Given that the percentage of deceased patients is similar in the two groups, the lower number of patients lost in the HLA-typed group is probably due to the higher motivation of these subjects to be regularly followed up. In the group of patients who did not agree to enter the study a higher proportion of males was lost to follow-up: the male/female ratio was 1.6 as compared with the 0.5 in the HLA-typed group. This observation explains the excess of women in the HLA-typed patients. Since the number of males is relatively small in this group, the higher than expected survival of males is most probably due to chance.

The observation that HLA locus B "inconsistency" correlates with poor prognosis in all subgroups controlled for sex and tumour thickness is convincing evidence of the importance of this "biological" prognostic criterion.

Further studies are needed to investigate the reason(s) of the lack of detectability of HLA locus B antigens on peripheral blood lymphocytes. We have no data at present to state whether the inconsistent detection of HLA-B antigens is due to an increased rate of cell membrane antigens shedding [18, 19] or to an alteration of the structure of major histocompatability complex molecules, according to the hypothesis of the alterated glycosylation [20] on class I subunit associations.

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