

INVESTIGATIVE REPORT

Cutaneous Squamoproliferative Lesions in Kidney Transplant Recipients: An Investigation of Specific Epstein-Barr Virus Expression

ANNIKA TERNESTEN-BRATTEL¹, CHRISTER KJELLSTRÖM¹, ANN-MARIE WENNBERG² and ANNE RICKSTEN³

Departments of ¹Pathology, ²Dermatology and ³Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg, Sweden

In a previous report we demonstrated Epstein-Barr virus expression in cutaneous squamous cell carcinomas from heart transplant recipients. In a comparative study, skin lesions from renal allograft recipients were investigated for the presence of Epstein-Barr virus. Thirty cutaneous squamoproliferative lesions from 10 kidney transplant recipients were examined for Epstein-Barr virus-specific gene expression. The techniques used for detection were polymerase chain reaction, *in situ* hybridization and immunohistochemistry. Epstein-Barr virus DNA was not detected by polymerase chain reaction in the neoplasias, and only single Epstein-Barr virus-positive carcinoma cells were shown by *in situ* hybridization in three cases of infiltrative squamous cell carcinomas. Immunohistochemistry for Epstein-Barr virus latent membrane protein 1 showed a negative result in all samples. These findings differ from our earlier investigations of cutaneous squamous cell carcinomas from heart transplant recipients where Epstein-Barr virus expressions were common. This may indicate that the part Epstein-Barr virus plays in the development of post-transplant, cutaneous squamoproliferative disorders is related to type of organ transplantation and/or grade of immunosuppression. **Key words:** Epstein-Barr virus; immunosuppression; skin cancer; transplant recipients.

(Accepted October 8, 2002.)

Acta Derm Venereol 2003; 83: 14–17.

Annika Ternesten-Brattel, Department of Pathology, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden. E-mail: annika.ternesten@path.gu.se

Immunosuppression after solid organ transplantation is often complicated by the development of different types of neoplasias. The most common type of malignant tumour in organ transplant recipients is non-melanoma skin cancer (NMSC), which is well documented in kidney transplant patients (1, 2). Recent studies have indicated that the risk of NMSC differs depending on type of organ transplantation and immunosuppressive regimen (3, 4). In general, transplant recipients show up to a 100-fold increased risk of NMSC (5).

Epstein-Barr virus (EBV) is closely related with tumour development in immunosuppressed patients, as has been stated in different investigations. This herpes virus infects most of the world's population and is normally a silent infection establishing lifelong latency. EBV has been detected in most post-transplant lymphoproliferative disorders (PTLD), a type of lymphoid neoplasm commonly encountered in organ transplant recipients (6). Excepting PTLD, EBV has been reported in oral hairy leucoplakia, an epithelial tumour arising in immunocompromised patients (7). Some studies have also implicated EBV in the development of squamous cell carcinoma (SCC) of the lip as well as the skin in immunosuppressed organ transplant recipients (8, 9).

In a previous study, we investigated cutaneous SCC in a Swedish group of heart transplant recipients for the presence of EBV DNA and EBV-specific products. Our results indicated that EBV could influence the development of skin tumours in this group of patients (9). In other studies, the results have been contradictory, with no evidence of EBV in cutaneous squamoproliferative lesions from organ transplant patients (10, 11).

The aim of this study was to investigate premalignant and malignant squamoproliferative skin neoplasms from a group of immunosuppressed renal allograft recipients for the prevalence of EBV DNA, RNA and protein.

MATERIAL AND METHODS

Patients and histopathological evaluation of the tumour material

The tumour material was randomly retrieved from the storage files of the Department of Pathology, Sahlgrenska University Hospital. A list of cutaneous squamoepithelial neoplasias, diagnosed in patients known to be immunosuppressed kidney transplant recipients, was generated from our database. From this material, 10 actinic keratoses (AK), 10 squamous cell carcinomas *in situ* (SCCIS) and 10 infiltrative SCC, high to low grade, were reviewed to confirm the diagnosis. These neoplasias were gathered from 10 kidney transplant recipients who had developed their first squamocellular neoplasms between 4 and 10 years after their first transplantation. The patient's age at transplantation ranged from 17 to 69 years with a mean of 44.2 years. All tumours were located in

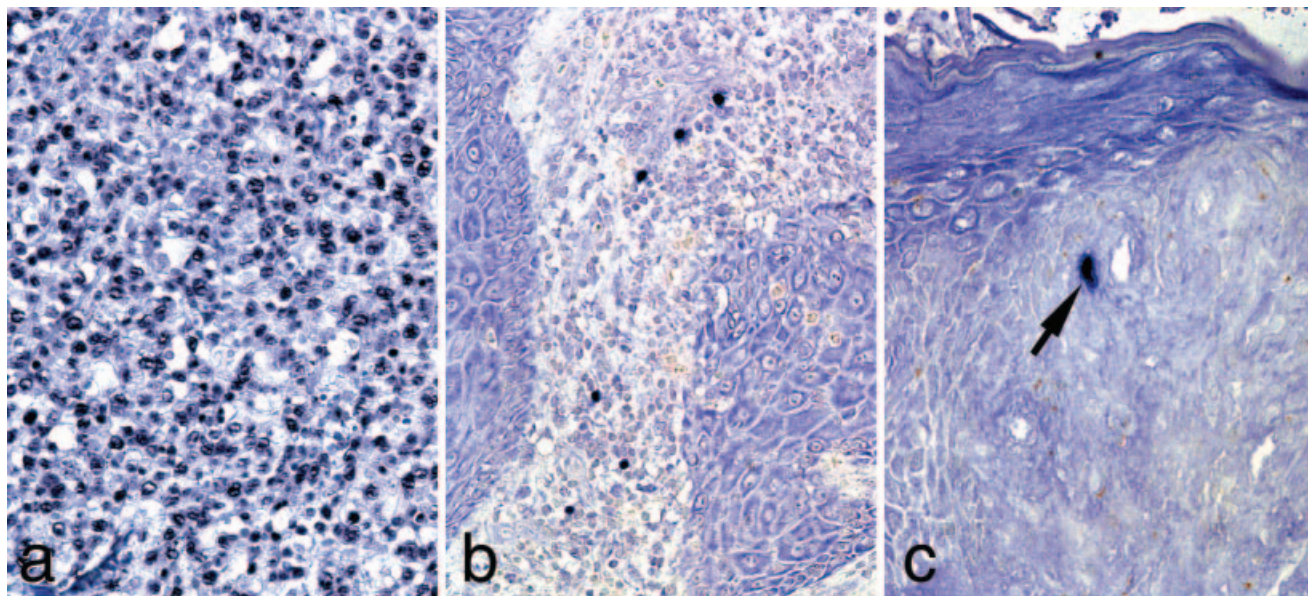


Fig. 1. (a) *In situ* hybridization for Epstein-Barr virus-encoded RNA (EBER), positive control. (b) Internal control, EBER-positive lymphoid cells in the stroma of a negative, low-grade squamous cell carcinoma; patient no. 5. (c) Single EBER-positive carcinoma cell in the superficial part of a low-grade squamous cell carcinoma; patient no. 4.

sun-exposed skin. The tumour material had been routinely processed, i.e. fixed in formalin and embedded in paraffin. Sections 5- μ m thick were deparaffinized, dehydrated, air-dried and routine stained with haematoxylin-eosin before they were re-evaluated. Tumour blocks with the least inflammation were selected for further investigation. In six tumour cases, paraffin blocks from tumour-free resection margins were available. These resection margins were used as internal controls and were subjected to the same analysis as the tumour material. Paraffin blocks from an EBV-associated post-transplant non-Hodgkin B-cell lymphoma were used as a positive control.

Polymerase chain reaction

Sections of 7 μ m, corresponding to a tissue area of 0.5 cm², were cut in a microtome with disposable blades from the same paraffin-embedded material as was used for the histopathological evaluation. The sections were taken from the superficial part of the sample in order to concentrate on the epidermis and tumour cells. The tissue was then transferred under sterile conditions to a 1.7-ml microcentrifuge tube with 1 ml xylene. Two treatments with xylene were used for deparaffinization. The samples were pelleted, washed in

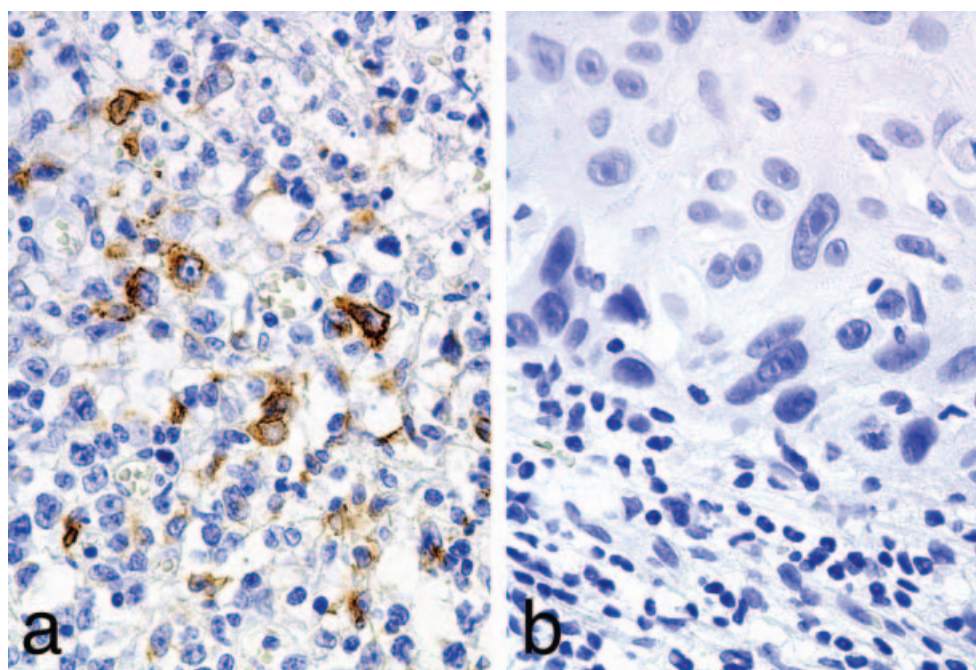


Fig. 2. (a) Immunohistochemistry for latent membrane protein 1 (LMP1), positive control. (b) LMP1 negative high-grade squamous cell carcinoma and stromal lymphoid cells, patient no. 7.

absolute ethanol and dried under vacuum. The material was then proteinase K digested (100 µl digestion buffer containing 0.20 mg/ml proteinase K, 50 mM Tris buffer, pH 8.5, 1 mM EDTA and 0.5% Tween 20) at 37°C overnight. Proteinase K was inactivated by incubation of the samples at 95°C. The DNA extracts were stored at -70°C until polymerase chain reaction (PCR) analysis.

EBV-analysis: PCR amplification was carried out using the same procedures as described in detail elsewhere (12). The primers used in the nested PCR assay were selected from the repeated sequences of the BAM H1 W fragment of the prototype EBV strain, B95-8. The outer primer set, designated W1 and W2, amplified a 275-bp segment, whereas the nested primer set, designated W3 and W4, amplified a 192-bp-long segment. In all the PCR assays, a positive EBV DNA control from P3HR-1 cells was used. The positive control was used in a low concentration (10 ng) and added as the last sample.

β-globin analysis: All samples were amplified with β-globin primers to exclude false-negative results (13).

In situ hybridization for Epstein-Barr virus encoded RNA

Five FITCH-labelled 30-base oligonucleotides, complementary to EBV-encoded RNA (EBER) 1 and 2 transcripts (Dakopatts, Stockholm, Sweden), were used for EBV RNA *in situ* hybridization (ISH). Paraffin sections (5 µm) from the same blocks as used for PCR were deparaffinized, dehydrated, digested with proteinase K and hybridized overnight at 37°C. Detection was accomplished with an anti-FITCH monoclonal antibody conjugated with alkaline phosphatase and visualized using NBT/BCIP. Tissue sections from an EBV-positive PTLD served as control (Fig. 1a). In some of the samples, there were EBER-positive lymphocytes, which served as an internal control (Fig. 1b). The samples were tested with an oligo-dT probe as control for integrity of the mRNA.

Immunohistochemistry for latent membrane protein 1

Sections from the paraffin blocks were deparaffinized, dehydrated and washed in phosphate-buffered saline (PBS). The streptavidin biotin complex technique was employed, using EBV-latent membrane protein 1 (LMP1) monoclonal antibodies (cocktail CS 1-4 Dakopatts, Sweden). Because of difficulties interpreting some areas rich in melanin pigmentation, some of the cases were re-examined with a different cromogen, alkali phosphatase. The case of EBV-positive PTLD was used as positive control (Fig. 2a).

Table I. Patients and material investigated

Patient no./sex	Age at transplantation	Tumours and control samples
1/M	69	1 AK, 1 SCCIS, 1 low-grade SCC
2/M	17	1 AK, 1 SCCIS, 1 low-grade SCC
3/M	48	1 AK, 2 SCCIS
4/M	33	1 AK, 1 SCCIS, 1 low-grade SCC
5/F	25	1 AK, 1 SCCIS, 1 low-grade SCC, 1 RM
6/M	32	1 AK, 1 SCCIS, 2 high-grade SCC, 2 RM
7/M	61	1 AK, 1 SCCIS, 1 high-grade SCC
8/M	42	1 AK, 1 SCCIS, 1 low-grade SCC, 1 RM
9/M	68	1 AK, 1 SCCIS, 1 low-grade SCC, 1 RM
10/M	47	1 AK, 1 SCCIS, 1 RM

M= male, F= female, AK= actinic keratosis, SCCIS= squamous cell carcinoma in situ, SCC= infiltrative squamous cell carcinoma, RM= resection margin.

RESULTS

A total of 30 pre-malignant and malignant cutaneous squamocellular neoplasias, excised from 10 immuno-suppressed kidney transplant recipients, were identified from our database. The group consisted of one woman and nine men. The tumours comprised of 10 AKs, 10 SCCISs and 10 infiltrative SCCs. The patients and tumour tissues studied are given in Table I.

The nested PCR used on DNA extractions from the paraffin-embedded material showed no EBV DNA in any of the investigated tumours or resection margins. A positive EBV DNA control from P3HR-1 cells was included in all the PCR assays, and β-globin primers were used to confirm the integrity of the DNA preparations.

ISH for EBER showed positive lymphoid cells in 12 of the investigated neoplasias (Fig. 1b). EBER staining was also positive in single carcinoma cells in three of the tumours (Fig. 1c). The EBER-positive carcinoma cells were detected in three cases of infiltrative SCC. All the remaining tumours and resection margins failed to show any EBER signal.

Detection with immunohistochemistry for the EBV-encoded protein LMP1 showed a negative result in the tumours as well as stromal lymphocytes (Fig. 2b) and resection margins.

DISCUSSION

We have investigated 30 squamoproliferative lesions from 10 renal allograft recipients for the presence of EBV genomes and EBV-specific gene expression by using three different techniques: PCR, ISH and immunohistochemistry.

Using PCR, no EBV DNA could be detected in the investigated tumours. The negative results were obtained in the presence of a strong positive control with each PCR running. False-negative results were excluded by a positive β-globin PCR in all our samples. The control materials, six resection margins from six different tumours, were also negative for EBV DNA by PCR.

The investigation was continued with detection of EBER by ISH. There were only single positive carcinoma cells in 3 of the 30 investigated tumours (Fig. 1c). These three tumours were all cases of infiltrative SCC and were isolated from three different patients. Twelve of the investigated tumours showed EBER-positive infiltrating lymphocytes (Fig. 1b). EBER could not be detected in the control material, which consisted of six resection margins. Immunohistochemistry for LMP1, an EBV-specific latent protein, showed a negative result in the investigated tumours (Fig. 2b) as well as the resection margins.

In two previous studies (10, 11) of NMSCs from organ transplant recipients, part of our investigative technique was used; namely ISH for EBER. Neither

study was able to identify any EBV gene products. The organ transplant patients in those studies were a heterogeneous group, with a mixture of heart, heart/lung and lung/lung as well as kidney organ transplant recipients.

In an earlier report (9), we investigated 15 NMSC from a homogeneous group of cardiac allograft recipients. These tumours were investigated with the same three methods as in the present study of renal allograft recipients. We detected EBV DNA by PCR in 10 of the tumours and 7 of these also expressed LMP1 and/or EBER.

The results in our present study of renal allograft recipients differ from our earlier report of NMSC in cardiac allograft recipient. There are similarities between these two groups of patients in terms of the clinical setting and in type of tumour development, but there are also obvious differences.

The number of patients receiving organ transplants is increasing. In order to avoid rejection, patients are put on immunosuppressive medication. When organ transplantation started, immunosuppressive treatment consisted of corticosteroids and azathioprine. More recently, cyclosporine and other immunomodulators have been added, making other types of organ transplantation possible. These drugs have several side effects, including an increase in the incidence of premalignant and malignant skin disorders, mainly NMSC.

For organ-transplanted patients with cutaneous SCC, the combination of corticosteroids, azathioprine and cyclosporine seems to increase the risk of NMSC more than the combination of only steroids and azathioprine (4, 14).

Patients with kidney transplants generally receive a less pronounced immunosuppression compared with heart transplant recipients who often receive triple drug medication. Consequently, heart transplant patients are at greater risk of developing SCC than kidney transplant recipients (3, 4). The progression of the lesions is more rapid in heart-transplanted patients, together with a higher risk of metastases and fatal outcome. New types of immunosuppressive regimes in this group of patients have also been correlated to a higher incidence of EBV-associated PTLD (15).

Our findings indicate a closer association between EBV and cutaneous squamoproliferative lesions in heart transplant patients compared to kidney transplant patients. Further studies of EBV latent gene expression and latent gene function in these malignancies, together with clinical observations, are needed for a better understanding of the role of EBV in malignant transformation of epidermal cells in immunocompromised patients.

ACKNOWLEDGEMENTS

This study was supported by grants from the Göteborg Medical Association, the Swedish Cancer Society, Sahlgrenska

University Hospital (LUA research), Assar Gabrielsson's Fund as well as Dr Gelin's Memorial Fund and the renal association in Sweden (Njursjukas förening/RNJ). We thank Tina Ekviden for her excellent technical assistance.

REFERENCES

1. London NJ, Farmery SM, Will EJ, Davison AM, Lodge JP. Risk of neoplasia in renal transplant patients. *Lancet* 1995; 346: 403–406.
2. Blohmé I, Larkö O. Skin lesions in renal transplant patients after 10 to 23 years of immunosuppressive therapy. *Acta Derm Venereol* 1990; 70: 491–494.
3. Euvrard S, Kanitakis J, Pouteil-Noble C, Dureau G, Touraine JL, Faure M, et al. Comparative epidemiological study of premalignant and malignant epithelial lesions developing after kidney and heart transplantation. *J Am Acad Dermatol* 1995; 33: 222–229.
4. Jensen P, Hansen S, Møller B, Leivestad T, Pfeffer P, Geiran O, et al. Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. *J Am Acad Dermatol* 1999; 40: 177–186.
5. Lindelöf B, Sigurgeirsson B, Gabel H, Stern RS. Incidence of skin cancer in 5356 patients following organ transplantation. *Br J Dermatol* 2000; 143: 513–519.
6. Bernstein D, Baum D, Berry G, Dahl G, Weiss L, Starnes VA, et al. Neoplastic disorders after pediatric heart transplantation. *Circulation* 1993; 88: 230–237.
7. Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Contant MA, Petersen V, et al. Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. *N Engl J Med* 1985; 313: 1564–1571.
8. Thomas DW, Ramsahoye B, Jasani B, Lim SH. Epstein-Barr virus in squamous cell carcinoma after renal transplantation. *Transplantation* 1995; 60: 390–392.
9. Ternesten-Bratell A, Kjellström C, Ricksten A. Specific expression of Epstein-Barr virus in cutaneous squamous cell carcinomas from heart transplant recipients. *Transplantation* 1998; 66: 1524–1529.
10. Huston BM, Maia DM. Absence of latent Epstein-Barr virus in cutaneous squamoproliferative lesions after organ transplantation. *Mod Pathol* 1997; 10: 1188–1193.
11. Kohler S, Kamel OW, Chang PP, Smoller BR. Absence of human herpes virus 8 and Epstein-Barr virus genome sequences in cutaneous epithelial neoplasms arising in immunosuppressed organ-transplant patients. *J Cutan Pathol* 1997; 24: 559–563.
12. Landgren M, Kyllerman M, Bergström T, Dotevall L, Ljungström L, Ricksten A. Diagnosis of Epstein-Barr virus-induced central nervous system infections by DNA amplification from cerebrospinal fluid. *Ann Neurol* 1994; 35: 631–635.
13. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985; 230: 1350–1354.
14. Glover MT, Deeks JJ, Raftery MJ, Cunningham J, Leigh IM. Immunosuppression and risk of non-melanoma skin cancer in renal transplant recipients. *Lancet* 1997; 349: 398.
15. Swinnen LJ, Costanzo-Nordin MR, Fisher SG, O'Sullivan EJ, Johnson MR, Heroux AL, et al. Increased incidence of lymphoproliferative disorder after immunosuppression with monoclonal antibody OKT3 in cardiac-transplant recipients. *N Engl J Med* 1990; 323: 1723–1728.