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Nasopharyngeal Carcinoma: Histopathological Types and Association with Epstein–Barr Virus

Ulla Hørding, Henrik Winther Nielsen, Henrik Albeck and Søren Daugaard

The polymerase chain reaction was used to examine paraffin-embedded tissues of 37 nasopharyngeal carcinomas (NPC) for Epstein–Barr virus (EBV) genomic sequences. EBV DNA was found in 2/14 keratinising squamous cell (WHO 1) carcinomas and in all of 23 non-keratinising and undifferentiated (WHO 2 and 3) NPC. The study confirms the infrequent association of keratinising NPC and EBV, in contrast with the 100% association of the less differentiated NPCs and the virus. The results may indicate a different carcinogenesis for the WHO 1 NPC subtype.
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INTRODUCTION

EPSTEIN–BARR virus (EBV), the causative agent of infectious mononucleosis, is also known for its consistent association with Burkitt's lymphoma in Africa and with nasopharyngeal carcinoma (NPC) [1, 2]. The latter is an uncommon tumour in most of the world, but of high prevalence in southeast China, east central Africa and in the Inuit of the Arctic regions.

According to the WHO classification [3], NPC may be classified into three categories. Keratinising squamous cell carcinomas, WHO 1, exhibit definite evidence of squamous differentiation with the presence of intercellular bridges and/or keratinisation over most of their extent. Non-keratinising WHO 2 carcinomas retain epithelial growth patterns, but squamous differentiation is not evident on light microscopy. Undifferentiated carcinomas (WHO 3) do not show any keratinisation and have a syncytial appearance. Some WHO 2 and 3 NPC contain numerous lymphocytes dispersed among the tumour cells, hence the term "lymphoepithelioma." The undifferentiated nasopharyngeal carcinoma is the type most frequently encountered.

According to the literature, the association of type 2 and 3 NPC and EBV seems to be almost at 100%. There is, however, conflicting evidence as to whether the keratinising NPC are also EBV-associated [4–9]. Besides, they differ from the undifferentiated NPC by a more even geographical distribution. Clinically, the keratinising tumours lead to a significantly worse outcome, in part, perhaps, because of their lesser radiosensitivity [10–12].

Correspondence to U. Hørding.
U. Hørding is at the Department of Gynaecology and Obstetrics, H.W. Nielson is at the Department of Pathology, H. Albeck is at the Department of Otorhinolaryngology, 2/4501 Rigshospitalet, University Hospital, Blegdamsvej 9, DK-2100 Copenhagen; and S. Daugaard is at the Department of Pathology, Frederiksberg Hospital, Copenhagen, Denmark.
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The very sensitive method of the polymerase chain reaction (PCR) has made possible the examination of archival tissues from rare tumours, like keratinising squamous cell carcinomas of the nasopharynx. We have used the method to examine the paraffin-embedded tissues of 14 keratinising carcinomas for EBV. For comparison, a group of undifferentiated NPC were examined with the same technique.

MATERIALS AND METHODS

Tissues

Buffered formalin-fixed, paraffin-embedded tissues from 37 NPC were retrieved from the archives of the Department of Pathology at Rigshospitalet, University Hospital of Copenhagen. The specimens had been obtained during the 26-year-period from 1965 to 1991 from patients referred from Greenland and from the eastern region of Denmark. They were reviewed independently by two pathologists (HWN and SD) without knowledge of viral diagnosis, and classified according to the WHO recommendations. It was decided to consider non-keratinising and undifferentiated squamous cell carcinomas (WHO type 2 and 3) as one group. Only tumours of unequivocal nasopharyngeal origin were included in the study. There were 14 keratinising squamous cell carcinomas and 23 carcinomas of non-keratinising and undifferentiated type. All of the keratinising and eight of the less differentiated/undifferentiated NPC belonged to Danish patients, and the remaining 15 tumours were from Greenlandic (Inuit) patients.

Several 7 μm sections were cut from the tissue block containing the most representative pathological changes in each case, and the first and the last sections were stained with haematoxylin and eosin for control of the specimen.

Sample preparation

The technique has been described in detail in a previous paper [13]. In brief, a 7 μm section was placed in a tube and deparaffinised in xylene, washed twice in ethanol, air-dried, then subjected to DNA extraction for 3 h at 55°C in a 100 μl buffer solution containing proteinase K and the detergent

Tween-20. After protease inactivation by heating, the sample was centrifuged briefly and a 15 µl aliquot of the supernatant was subjected to PCR (polymerase chain reaction).

The primers

PCR amplification was carried out with either of two sets of oligo primers: one set specific for a 110 bp sequence of the 10 or 11 times reiterated *Bam*H1 W region of the EBV genome [14, 15], and one set specific for a 110 bp sequence of the human β-globin genome [16]. Amplification with the latter set of primers was included to ensure that the DNA of the tissue was still of sufficient quality to permit viral diagnosis by PCR.

PCR Amplification

Fifteen microlitres of freshly extracted tissue DNA was added to the PCR reaction buffer to a total volume of 100 µl containing 100 µmol/l of each of the four deoxynucleotide triphosphates, 10 mmol/l Tris–HCl at pH 8.3, 50 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 0.01% gelatin, 50 pmol each of 20-mer synthetic oligo DNA primers, and 2 units of *Taq* polymerase (Amplitaq®, Cetus Corp.). The samples were overlaid with two drops of mineral oil to prevent evaporation and subjected to 40 cycles of amplification in a DNA thermal cycler (Perkin-Elmer-Cetus Instruments). Each cycle consisted of a 96°C denaturation step of 20 s, a 56°C primer annealing step of 20 s, and a 72°C primer extension step of 35 s. In the first cycle the denaturation step was extended to 2 min at 94°C, and in the final cycle the primer extension step was prolonged to 10 min.

Each set of 14 reactions included one positive and one negative control. In reactions with EBV-specific primers, the controls consisted of EBV-positive DNA from about 30 formalin-fixed, paraffin-embedded Raji cells, and of EBV-negative DNA from sections of formalin-fixed, paraffin-embedded heart muscle. In reactions with primers specific for a part of the human genome, the positive control was DNA from heart muscle and the negative control consisted of PCR buffer only.

Dot-blot hybridisation

Following PCR, 10 µl of the amplification product was spotted onto each of two GeneScreen Plus membranes through a dot-blot apparatus (Hybridot®, Bethesda Laboratories), and fixed to the membranes by alkaline treatment. DNA–DNA hybridisation was performed in aqueous solution [17] over night using a ^{32}P 5′-end-labelled oligonucleotide probe specific to the internal portion of the amplified sequence. DNA hybridisation and subsequent washing of membranes was done at 49°C (T_m—10°C for the probe specific for the human β-globin genome) or at 62°C (T_m—11°C for the EBV probe). The dot blots were subjected to autoradiography for about 2 h on an X-ray film with intensifying screens, then scored positive or negative by comparison with the control samples.

RESULTS

Amplification by PCR was successful in all of the tissues, as indicated by a positive signal upon hybridisation with the probe specific to the 110 bp amplification target of the human genome.

EBV genomic sequences were found in two (14%) of the 14 keratinising squamous cell carcinomas, and in all of the 23 non-keratinising and undifferentiated NPC. The two EBV-positive keratinising NPC were not morphologically different from the other tumours of that subtype. Hybridisation signals were strong in 11 undifferentiated NPC and moderate in the remainder of EBV-positive tissues.

DISCUSSION

The unique association of anaplastic nasopharyngeal carcinoma and EBV has been recognised for many years; according to some larger studies, the serology of patients with WHO 2 and 3 NPC may actually reflect the tumour burden and, conversely, patients with WHO 1 carcinomas have EBV serological profiles similar to those of control populations [5]. Molecular hybridisation studies [4, 6, 7, 9] have also demonstrated an almost 100% association of WHO 2 and 3 nasopharyngeal carcinoma and EBV. In the largest of these studies, which was based on *in situ* hybridisation, Niedobitek *et al.* [8] found only 68 of 77 WHO 3 NPC were EBV-positive; it is, however, possible that the DNA of some of the tissues examined was too damaged, by fixative and by time, to permit the viral diagnosis.

The association of EBV and keratinising NPC is much less clear. In the hitherto largest study by molecular hybridisation techniques, *in situ* hybridisation failed to show EBV in any of eight WHO 1 NPC [8]. On later examination of three of these tumours, however, the more sensitive PCR technique revealed one EBV-positive specimen. Raab-Traub *et al.* [6] reported EBV DNA in low but detectable numbers in all of five WHO 1 tumours (and EBV DNA in high numbers in all of 28 WHO 2 and 3 NPC). The higher EBV content in undifferentiated NPC, compared to EBV-positive keratinising tumours, has been confirmed to some degree in this study, and also in two other PCR-based studies [7, 9], in which EBV DNA was demonstrated in 5/7 and 3/5, respectively, of the keratinising NPC examined.

It is still under debate, whether all the three morphological subtypes of NPC are expressions of the same oncogenetic process in the nasopharyngeal mucosa. Much controversy in the published studies may have been caused by the fact, that it is not always possible for the pathologist to determine unequivocally, whether a tumour should be classified as WHO type 1 or type 2 NPC. Further, these tumours exhibit a high degree of heterogenicity, and a small biopsy taken at random from a partially invisible lesion may show the typical features of an NPC 1 tumour, although the rest of the tumour exhibits the pathological changes of NPC 2 or even 3.

The demonstration of EBV DNA in a few keratinising NPC in this and other studies based on tumour DNA preparations could be an admixture of EBV DNA from by-stander cells. Several studies have, however, shown that EBV is invariably present only in the tumour cells and not in the adjacent squamous epithelium [4, 8] or in infiltrating lymphocytes [18]. Lymphocyte infiltration is not a prominent feature in type 1 NPC, and the lymphocytes of the lymphoepithelioma are primarily T lymphocytes, not the EBV receptor-carrying B lymphocytes [19]. On the other hand, just a few EBV-carrying B lymphocytes escaped from the lymphatic tissues in proximity of the tumour region may, due to the extreme sensitivity of the PCR technique, be the cause of an apparently EBV-positive keratinising carcinoma.

In our opinion, the different epidemiology and the inconstant EBV association of the keratinising nasopharyngeal carcinoma indicate that this is a cancer type quite different from

the type 2 and 3 NPC. The pharyngeal mucosa seems to be the target tissue of EBV infection, and the undifferentiated keratinocytes of this epithelium have been shown to have EBV receptors [20, 21]. It is possible that the undifferentiated, but not the keratinising, NPC originate from these cells. Alternatively, all NPC have a common origin, and the virus may actually direct tumour morphogenesis. This compares to cervical carcinoma, in which two clinically and morphologically distinct tumour types are associated with each its specific, and putatively oncogenic, papillomavirus, even though both tumours apparently arise from the same cells [13].

In conclusion, although the consistent association of EBV and the undifferentiated nasopharyngeal carcinoma does not prove a causative role for the virus in this particular cancer, the regular coexistence of the virus does indicate a carcinogenesis different from that of the keratinising carcinoma.

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