Identification of fatal outcome in a childhood nasopharyngeal carcinoma patient by protein expression profiling

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Abstract. Nasopharyngeal carcinoma (NPC) is a rare disease in children with good prognosis and high cure rate. Nevertheless, certain patients have an unfavorable prognosis due to development of refractory NPC that is unresponsive to any therapeutic strategies. The current study studies a case of a 17 years-old female with non-keratinizing NPC type IIb (T2N0M0), who passed away as a consequence of resistance to chemo-, radio- and β-interferon therapy, and to an allogenic stem cell transplantation. In order to identify factors that lead to treatment failure and fatal outcome, immunohistochemical analyses of different tumor biomarkers and hierarchical cluster analysis were performed and compared with those of eight other patients with NPC who experienced complete remission following conventional therapy. Hierarchical cluster analysis of the immunohistochemical results clearly demonstrated that staining for immunological factors (CD4, CD8 and CD56) distinguished this patient from the others. To further investigate a potential role of the immune system, lymphocytic infiltration was assessed in tumor tissue by evaluation of hematoxylin and eosin-stained tumor sections. Indeed, no tumor infiltrating lymphocytes (TILs) were observed in this NPC case, while 7 out of 8 of the other NPC samples contained variable TIL amounts. The view that immunodeficiency of the patient may be a factor in the fatal outcome of treatment is supported by the fact that this patient with NPC was not positive for Epstein-Barr virus markers and also infected by several other viruses and fungi (herpes simplex virus, human herpes virus 6, Varicella zoster virus, and Candida). In conclusion, the investigation of rare NPC cases with poor prognosis may provide an improved understanding of the molecular mechanisms involved in refractory tumors and identification of novel potential therapeutic targets for NPC in the future.

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor that arises from the epithelial lining of the nasopharynx, and it is considered the most common cancer of the head and neck (1). NPC has a distinct racial, sex and geographical distribution with a multifactorial etiology. China, Southeast Asia and North Africa have the highest prevalence worldwide. According to World Health Organization (WHO) estimates, the Western Pacific and Southeast Asia regions accounted for ~77% of all cases internationally. Furthermore, NPC is particularly more frequent in males (sex ratio 2.3:1) and certain ethnicities, such as Chinese (2,3). The occurrence of NPC in childhood is rare. It has been estimated that 5% of childhood primary tumors arise in the head and neck area, while NPC only constitutes 2% of head and neck tumors in children (4,5).

Multiple etiological factors have been reported to be associated with the risk of developing of NPC, including genetics [certain human leukocyte antigen (HLA) types], environmental factors (nitrosamines and tobacco) and viral infection [Epstein Barr virus (EBV)] (6,7). The WHO has classified the NPC into three types, based on histopathological criteria: Keratinizing squamous cell carcinoma (type 1), non-keratinizing carcinoma (type 2) and undifferentiated carcinoma (type 3). It has been reported that these types are geographically diversified. In Southern China, where NPC is highly prevalent, nearly all cases are of the undifferentiated type, whereas in the USA, where NPC is rare, ~20% cases are the keratinizing type (8).
Although EBV has long been associated with nasopharyngeal carcinogenesis, several studies have reported an association of oncogenic human papillomaviruses (HPV) with a sub-group of NPCs (9-12). Furthermore, HPV is considered as a leading cause of cervical carcinoma (13). Whilst HPV has been reported as one of the causative agents of NPC in adults, little is known about the implication of HPV in the pathogenesis of childhood NPC.

In the present study, a rare case of a patient with a low-risk NPC type IIb with T2N0M0, which was expected to have a good prognosis, is reported. Surprisingly, the tumor was refractory to therapy and even allogenic stem cell transplantation failed, which ultimately led to mortality. The poor prognosis was not predictable by standard clinicopathological parameters. Thus, the expression of a panel of biomarkers was analyzed by immunohistochemistry in formalin-fixed, paraffin-embedded (FFPE) tumor material from this patient and compared with biopsies from 8 other childhood NPC cases. All 9 patients were enrolled in the prospective multicenter study [Nasopharyngeal Carcinoma (NPC) 2003 German Society of Pediatric Oncology and Hematology/German Children's Oncology Group (NPC-2003-GPOH/DCOG)] (14,15). The biomarkers included human papilloma virus markers (E6, E7), immunological markers (CD4, CD8 and CD56), proliferation markers [Ki-67, 14-3-3β, eukaryotic translation initiation factor 3 subunit E (eIF3e)], tumor suppressors [p53, Wilms tumor 1 (WT1)], apoptosis markers [terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, p53, ATP-ADP translocase (AAC)] and other markers [glutathione S-transferase π1 (GSTP1), CD31, inducible nitric oxide synthetase (iNOS)].

Materials and methods

Patients. A 17 year-old female patient was diagnosed with a low-risk nasopharyngeal tumor at the Department for Pediatrics (Medical Center of the University of Tübingen, Tübingen, Germany) on March 16th, 2007. The histology was determined as non-keratinizing squamous epithelial carcinoma with lymphoid infiltration of type IIb with T2N0M0 stage. Therapy was performed according to NPC-2003-GPOH (14) and consisted of: i) Tumor irradiation including lymph vessels with 59.4 and 45 Gy, respectively; ii) two chemotherapy cycles and consisted of: i) Tumor irradiation including lymph vessels with 59.4 and 45 Gy, respectively; ii) two chemotherapy cycles with cisplatin and 5-fluorouracil in the first cycle and cisplatin, 5-fluorouracil and docetaxel in the second cycle.

A second refractory tumor was diagnosed on October 17th, 2008. The patient was treated with chemotherapy again (cisplatin, 5-fluorouracil and docetaxel). Recurrence was diagnosed on February 7th, 2008. Irradiation could not be performed, because of the large expansion of the tumor (intra-abdominal, retro-crusral, intra-thoracic, along the Truncus coeliacus and para-aortic). AS chemotherapy alone was not curative, allogenic stem cell transplantation was suggested. In parallel, there were signs of immunodeficiency, indicated by infection with Herpes simplex virus (HSV), human-HSV-6 (HHV-6), Varicella zoster virus (VZV) and Candida spp. in addition to EBV. Thus, allogenic stem cell transplantation from the HLA-identical 9 year-old brother of the patient was suggested as a potential curative treatment approach. As the brother was EBV-IgG-positive, it was expected that a T-cell-mediated antiviral response against EBV may be transferred by transplantation. The patient's tumor cells expressed EBV antigens. Therefore, an antiviral T-cell response may act as allos-versus-tumor response and, thus, as prophylaxis against renewed recurrence of the tumor.

Following agreement of the patient, the stem cell transplantation was performed. Conditioning took place at June 4th, 2008 with treosulfan and cyclophosphamide and was well tolerated except for a transient increase of liver transaminases and nausea. The allogenic bone marrow stem cells transferred on June 10th, 2008 contained 1.25x10^8 mononuclear cells/kg body weight (BW), 2.45x10^9 CD34-positive progenitor cells/kg BW, 0.70x10^9 AC133-positive progenitor cells/kg BW and 1,661.43x10^4 CD3-positive cells/kg BW. Antibiotic prophylaxis was performed with metronidazole (800 mg/day for 29 days) and cotrimoxazol (160 mg/day for 28 days), fungal prophylaxis with amphotericin B (150 mg/day for 29 days) and viral prophylaxis with acyclovir (2,400 mg/day for 35 days). Graft-versus-host disease was treated with cyclosporine (90 mg/day for 16 days), methotrexate (15 mg/day for 1 day) and prednisolone (100 mg/day for 5 days). Granulocyte colony stimulating factor growth factor was applied 11 times between day 4 and 14 after transplantation. The course of the transplantation was free of complications.

Toxicity (WHO grade IV) occurred in the aplastic phase (mucositis, nausea, transient increase of transaminases). Graft-versus-host disease of the skin (grade 1) appeared on day 13 after transplantation. The engraftment took place on June 24th, 2008 (day 14 after transplantation). Arterial hypertension was observed as side effect of drug therapy. The atelectasis of the left lower lobe was persisting. Recurrence following stem cell transplantation was diagnosed on October 17th, 2009 and the patient died on January 25th, 2009.

In addition to this problematic patient, 8 pediatric NPC patients (age range, 11-17 years at diagnosis; 5:3 male:female ratio) that achieved complete remission following treatment according to the NPC-2003-GPOH regimen were investigated. FFPE tumor biopsies from all patients were provided by Dr Rolf Mertens (University Hospital Aachen, Aachen, Germany) and analyzed for comparison with the aforementioned clinical case. The tumor tissues were fixed in 10% neutral-buffered formalin at room temperature for 24 h. These FFPE samples have been investigated within the frame of the NPC-2003-GPOH/DCOG study (14).

Ethical approval (reference no. EK034/03) for the use of tumor material for experimental purposes was obtained from the Ethics Committee of the University of Aachen (Aachen, Germany), and local ethical approval was obtained from the participating centers in Germany (14). Written informed consent was obtained from all patients.

Immunohistochemistry staining and evaluation. Commercially available antibodies were applied on paraffin-embedded tissue
Assessment of tumor-infiltrating lymphocytes (TILs). Histological hematoxylin and eosin (H&E) routine staining (16) was performed on tumor sections, in order to assess TILs. The cells were counted with panoramic viewer software (NuclearQuant; 3DHISTECH Ltd.) according to the recommendations of the International TILs Working Group 2015 (17): i) The percentage of TILs in the stromal tumor tissue within the borders of the invasive tumor has been determined. TILs in normal areas outside the tumor were not considered; ii) tumor areas with crush artifacts, necrosis, or regressive hyalinization were not evaluated; iii) only mononuclear, but not polymorphonuclear leukocytes were counted; iv) the average values of the full tumor area were assessed, rather than focusing on hotspots.

Hierarchical cluster analysis. In the present study, hierarchical cluster analysis was performed to group heterogeneous objects into clusters of homogeneous objects. All objects were assembled into a cluster tree (dendrogram). Thus, objects with tightly related features appear together, whereas the separation in the cluster tree increases with progressive dissimilarity. The merging of objects with similar features leads to formation of a cluster, the shortest the distance of the branch the closest degree of relatedness (18). Hierarchical clustering was conducted using WinSTAT software (R. Fitch Software, Cambridge, MA, USA) and the Ward method (19) was performed. Previously, cluster methods have been validated for gene expression profiling, identification of candidate genes for drug resistance and sensitivity and for understanding molecular biology of cancer (20,21).

Results

Immunohistochemistry. An expression of a panel of biomarkers for tumor aggressiveness and progression was analyzed in a female patient with NPC type IIb (case 9). The expression patterns were compared with those of eight other patients with NPC (cases 1 to 8). Using immunohistochemistry, 15 different markers were detected, which may indicate tumor aggressiveness and may also predict tumor responsiveness to therapy. Representative immunostaining images of NPC case 9 and selected tumors of the other patients with NPC are depicted in Figs. 1-3; staining for human papilloma virus markers (E6, E7; Fig. 1), immunological markers (CD4, CD8 and CD56; Fig. 1), proliferation markers (Ki-67; 14-3-3-τ, eIF3e; Fig. 2), tumor suppressors (p53, WT1; Fig. 2), apoptosis markers (TUNEL assay, p53, AAC; Fig. 3) and other markers (GSTP1, CD31, iNOS; Fig. 3) is shown.

A general observation in most tumor tissues was that the expression pattern exhibited considerable heterogeneity; the number of stained cells and the staining intensities varied between different areas of the same tumor. For this reason, the immunostaining for all antibody and all tumors was quantified. Six representative areas were chosen from each tumor and the number of positively stained cells was counted using a digital slide scanning (Fig. 4).
subtypes of tumors, resistance or sensitivity of tumors towards anticancer treatments, survival chances of cancer patients, etc. Among the numerous clustering methods (e.g. topological interaction models, influence maps, physical regulatory maps, self-organizing maps, principal component analysis, etc.), supervised, hierarchical and aggregative techniques provide advantages for pharmacological questions in cancer biology and pharmacology, because of their flexibility, possibility to include biological knowledge with different weighting, and detection of higher-order relationships between clusters of profiles (22). Aggregative hierarchical clustering is a frequently used method to investigate gene expression signatures (23-25).

The quantified expression values obtained from immunohistochemistry were subjected to hierarchical cluster analysis in order to investigate, whether the protein expression profile of patient 9, who died following standard therapy and allogenic stem cell transplantation, could be differentiated from the profiles of the eight other NPC samples from patients that responded well to treatment.

If the results of all immunostainings were subjected to cluster analysis, a dendrogram with two clearly distinguishable branches appeared (Fig. 5A). However, case 9 did not appear as isolated branch separated from all other tumors, indicating that the panel of immunohistochemical parameters in its entirety was not able to identify this tumor.

Additionally, immunohistochemical staining markers were divided into groups consisting of immunological parameters [CD4 and CD8 as T-cell markers, and CD56 expression as marker of natural killer (NK) cells] and the remaining biomarkers were non-immunological markers. As shown in Fig. 5B and C, case 9 appeared isolated as single tumor in the dendrogram of the three immunological markers. The CD counts in case 9 were not the lowest of all samples, but the intermediate to low expression was different from the expression profile of these markers in the other NPC cases. Thus, cluster analysis separated case 9 as being distinct from the others when stained with immunological markers. The immunodeficient state of the patient was also validated by

Figure 1. Representative immunohistochemical staining for viral markers (HPV-E6 and E7) and immunological markers (CD4, CD8 and CD56) in pediatric nasopharyngeal carcinoma. HPV-E6 and HPV-E7 were expressed in the tumor cells, while CD4, CD8 and CD56 were expressed in tumor-infiltrating lymphocytes. Magnification, x40. HPV, human papilloma virus.
H&E-staining of TILs, where the lack of TILs in this patient became apparent. This was not observed in the dendrogram of all other non-immunological markers, indicating a potential specific role of the immune response in patient 9, which may differ from the other patients with NPC.

Assessment of TILs. To further validate that a deficient immune response may have contributed to the treatment outcome of NPC case 9, the percentage of TILs in tumor samples was evaluated using H&E-staining. TILs were red-stained and were counted with the same microscopic slide scanning procedure as used for immunohistochemistry, with the exception that NuclearQuant software was used for counting red-stained cells, rather than brown-stained cells. The bar chart in Fig. 6A shows that NPC case 9 and another tumor (case 8) did not contain TILs at all, whereas the other NPC cases contained variable fractions of TILs ranging from 0.06±0.05 to 43.7±15.3%. To further validate the specificity of TIL-staining by H&E, serial NPC sections stained with CD4 or CD8 antibodies were compared. As revealed by staining of serial sections of case 3 tumor, the TIL population identified by H&E-staining was also CD4- and CD8-positive in immunohistochemistry (Fig. 6B-D).

Discussion

NPC is a complex disease associated with EBV infection, environmental factors, and genetic aberrations (26). Although childhood NPC generally has a good prognosis, certain patients do not benefit from chemo- and radiotherapy (27). Although β-IFN-containing therapy regimens improve outcomes (13,14), cure rates of 100% are still achieved. The current study presents a case of a 17 year-old patient that could not be cured and finally died from the disease. This was surprising, as the patient was diagnosed with a low-risk type IIb tumor (T2N0M0), which was expected to have a good prognosis. Cases like these are still enigmatic, as treatment outcome or prognosis cannot be predicted by established...
clinical or pathoclinical parameters. Therefore, such cases deserve further attention and investigation.

To identify biomarkers that can predict rare cases of treatment non-responders, we immunohistochemical analyses were performed using a broad panel of antibodies against proteins involved in diverse cancer- and NPC-associated functions, including HPV, immune response, proliferation, tumor suppression, apoptosis and others. The quantified results were subjected to hierarchical cluster analysis, which is valuable for microarray-based mRNA-expression profiling, and also for protein expression data (28-30). The combination of three markers of immunological responses (CD4, CD8 and CD56) provided better separation of case 9 than when all markers were included.

CD4 and CD8 are key for the adoptive T-cell immune response. CD56 is a marker of NK cells, which are important for the innate immune system. Both categories of immune responses are involved in NPC. CD4+ and CD8+ T-cells (TILs) infiltrate NPC (28,29), and CD56+ NK cells act against EBV-infected cells (31). Animal experiments demonstrated that CD8-positive T-cells enhance immunity to EBV-associated malignancies; CD4+ and CD8+ T-cells act together synergistically in the antitumor response (32). In adult patients with NPC, it has been reported they have reduced numbers and function of cytotoxic T lymphocytes (CTLs) compared with normal subjects or long-term NPC survivors (33). In those patients, the cytolytic effects of NK cells were higher than that of healthy donors and NPC survivors (33). CTLs and NK cells compensate each other and are involved in immunity against NPC (33). Innate and adaptive immunity may exert antitumor responses in some, but not all patients with tumors. The reasons are not fully understood. CD4+ T-regulatory cells (Tregs) of the Th1-type and follicular helper cells were reported to be associated with favorable prognosis, whereas Th2-type Tregs were demonstrated to inhibit the antitumor immune responses (33,34). The presence of CD8+ TILs is significantly correlated with response to therapy and survival of patients (34). While TILs have been investigated by H&E-staining in lung
Figure 4. Quantification of immunohistochemical detection of biomarkers by Panoramic Viewer software (3DHISTECH) in nine pediatric nasopharyngeal carcinoma samples. Each six tumor areas free of necrotic and stromal parts were independently quantified per tumor sample. Values are presented as the mean ± standard deviation. HPV, human papilloma virus; eIF3e, eukaryotic translation initiation factor 3 subunit E; WT1, Wilms tumor 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling; AAC, ATP-ADP translocase; GSTP1, glutathione S-transferase π 1; iNOS, inducible nitric oxide synthetase.
The patient presented here was also refractory to chemo-, radio- and β-INF therapy, and to allogenic stem cell transplantation. It was hoped that IgG autoantibodies against EBV-associated antigens of the EBV-infected, but otherwise healthy brother could elicit a T-cell-mediated antiviral response (graft-versus-tumor response) against the tumor of the sister, and that this expected immune response may be transferred with the transplantation. Unfortunately, a sustainable tumor remission was not reached. The patient was severely immunodeficient. The addition of β-INF to the NPC-2003-GPOH treatment protocol was ineffective, although the beneficial effect of β-INF for the outcome of patients with NPC has been convincingly demonstrated by several clinical trials (13,14). Another indicator of the immunodeficient state of the patient was the infection with several other viruses and fungi in addition to EBV, including HSV, HHV-6, VZV and Candida. Although immunotherapeutic approaches may be attractive alternatives for non-responders to conventional treatments, immunodeficiency represents a resistance mechanism to immunostimulatory strategies, as indicated by the patient presented in this study.

The rationale to investigate the other biomarkers was their prognostic value for NPC reported in the literature. Although these markers were not able to discriminate case 9 from the other NPC cases, they are valuable markers to describe the aggressiveness of larger cohorts of NPC and other tumor types.

AAC staining was strong in all tumors, whereas, p53 detection was minimal. AAC is a mitochondrial protein that facilitates the exchange of ADP and ATP across the inner mitochondrial membrane and has an essential role in cellular energy metabolism. AAC is involved in metabolic adaptation during tumor development (Warburg effect) (39) causing apoptosis resistance in cancer cells. This supports the observation of low rates of apoptosis in the current study (TUNEL assay). It is not surprising that wild-type p53 was not highly detected, if the high AAC expression and low apoptosis rates are taken into consideration. Wild-type p53 is a nuclear phosphoprotein that triggers apoptosis or cell cycle arrest under cellular stress conditions (40-42). TP53 gene mutations are the most common genetic feature of tumors, identified in >50% of tumors (49,50). Loss of p53 function is associated with poor prognosis and drug resistance (51,52).

The HPV-E6 protein expression was high in almost all NPC biopsies investigated. HPV-E6 expression results from the integration HPV genomes into host chromosomes. HPV-E6 binds to p53 and promotes p53 degradation through the ubiquitin-proteasome pathway (43) and the p53 levels decrease (44). This observation coincides with the findings in NPC biopsies in the current study. The HPV-E7 protein was expressed in certain patients in the present study. HPV-E7 targets retinoblastoma-associated protein, pRb, which negatively regulates G1/S and G2/M cell cycle transitions (45).

Ki-67 is expressed during all cell cycle phases (G1, S, G2 and M) (46). It is a marker of cell proliferation and poor prognosis (47,48). Ki-67 was detected in almost all of our NPC specimens. The clinical significance of Ki-67 in patients with NPC has been reported previously (49).

Eukaryotic translation initiation factors (eIFs) are involved in the initiation of translation. The eIF3 complex binds to 40S ribosomal subunits and promotes the binding of methionyl-tRNA
and mRNA to form the 40S initiation complex (50). The eIF3a is involved in cell cycle progression (51) and has a role in tumorigenesis, re-sensitization to chemotherapeutics and improved prognosis (52). In the current study, the expression of eIF3e was detected in only 20% of NPC cases.

Nitric oxide (NO) is an inflammatory mediator and contributes to the inhibition of tumor suppressor functions and DNA repair, activation of oncogenes, angiogenesis, and metastasis (53-55). iNOS generates high amounts of NO over prolonged periods (63,64). The overexpression of iNOS is associated with high apoptosis rates, whereas low iNOS expression is associated with increased incidences of local tumor recurrence and metastasis following radiotherapy in patients with NPC (56). Tumor-derived iNOS is a pro-angiogenic factor and has been strongly implicated in angiogenesis via upregulation of vascular endothelial growth factor (57). In the present study, iNOS and CD31 (blood vessel marker) were observed in ~1/3 of patients.

In conclusion, childhood NPC has an excellent prognosis (13,14). However, as not all children are cured, research efforts have to be undertaken to improve current therapy options. The current study presented a patient with non-keratinizing NPC type IIb (T2N0M0), that was resistant to chemo-, radio- and β-IFN therapy, and to allogenic stem cell transplantation. Immunohistochemistry was performed to identify factors distinguishing this patient from a panel of other patients with NPC, who experienced complete remission following conventional therapy. By hierarchical cluster analysis, detection of immunological factors (CD4, CD8 and CD56) separated this patient from the others. The tumor in this patient recurred following β-IFN therapy, and EBV-directed autoantibodies of the HLA-identical brother did not provoke a graft-versus-tumor response upon allogenic stem cell transplantation. This and several concomitant infections indicated severe immunodeficiency as factor contributing to the fatal outcome. The analysis of more rare cases like this one may help to further improve treatment success of refractory childhood NPC in the future.

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Availability of data and materials

The data that support the findings of this study are available from Department of Pediatric and Adolescent Medicine, University Hospital Aachen (Aachen, Germany) and Department of Paediatric Haematology/Oncology, Children’s University Hospital (Tübingen, Germany), but restrictions apply to the availability of these data, which were used under patients’ consent for the current study, and so are not publicly available. Data are however available from the Department of Pediatric and Adolescent Medicine, University Hospital Aachen, and Department of Paediatric Haematology/Oncology, Children’s University Hospital.

Authors’ contributions

TE, RM and RH designed the study; MEMS performed the immunostaining and wrote the manuscript; RM and RH provided the material and treated the patients; TE performed the statistical analysis, wrote the paper, supervised the work and provided the facilities for the study. All authors read the manuscript and approved the final version.

Ethics approval and consent to participate

Ethical approval (reference number: EK034/03) for the use of tumor material for experimental purposes was obtained from the Ethics Committee of the University of Aachen (Aachen, Germany) on May 19th, 2003, and local ethical approval was obtained from the participating centers in Germany (14). Written informed consent for experimental work was obtained from all patients.

Consent for publication

Written informed consent for publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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