

ANATOMICAL PATHOLOGY / VIROLOGY

Diabetes can increase the prevalence of EBV infection and worsen the prognosis of nasopharyngeal carcinoma

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Summary

Epstein–Barr virus (EBV) infection is a primary oncogenic factor of nasopharyngeal carcinoma (NPC) that elicits epithelial–mesenchymal transition (EMT). Although diabetic patients are more susceptible to various infectious diseases, the pathological association with virus-related NPC has not yet been clarified. Herein, we evaluated the influence of diabetes on the clinicopathological changes of 70 patients with NPC. Disease-specific survival (DSS) modified by viral infection was also analysed. The proportion of NPC patients with diabetes was 32.9% (23/70 cases), and 91.3% (21/23 cases) were infected with EBV detected by EBER-I *in situ* hybridisation. NPC with diabetes showed an effect on EMT evaluated by immunostaining for E-cadherin and vimentin, which was correlated with HbA1c levels. Receiver operating characteristic (ROC) curve analysis determined a HbA1c level of 6.5% as the cut-off value for primary disease death at 2 years [area under the curve (AUC) 0.76; sensitivity 0.64; and specificity 0.81]. High HbA1c levels ($\geq 6.5\%$) significantly increased the number of lymph node metastases in NPC compared to low HbA1c levels ($< 6.5\%$, $p < 0.01$). Diabetic NPC patients had a significantly poorer prognosis than all non-diabetic patients (DSS, 72 months vs not reached, $p < 0.05$). Diabetic EBV-positive NPC patients had a significantly poorer prognosis than non-diabetic EBV-positive patients (DSS, 35 months vs not reached, $p < 0.01$). Multivariate analysis using the Cox proportional hazards model also suggested that HbA1c $\geq 6.5\%$ was a significant factor in poor prognosis, with a hazard ratio of 6.84 ($p < 0.05$). Collectively, our results revealed for the first time a high prevalence of EBV infection, poor prognosis and the importance of proper glycaemic control in diabetic NPC patients.

Key words: Nasopharyngeal carcinoma; type 2 diabetes; Epstein–Barr virus; human papillomavirus; epithelial–mesenchymal transition.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a carcinoma arising from the nasopharyngeal mucosal lining and is particularly prevalent in east and southeast Asia.¹ Its incidence has declined, and mortality has been reduced substantially.² On the other hand, its tumourigenesis is more complicated because multiple factors related to environmental factors are increased and contribute to the development of NPC, reflecting recent Western-like lifestyle changes in Japan.³

The importance of tumour-related viruses in pharyngeal cancer has increased in recent years.² Human papillomavirus (HPV) causes pharyngeal cancer including some NPC,⁴ and Epstein–Barr virus (EBV) is a major carcinogen of NPC.⁵ Compared to non-virus-related pharyngeal cancer, all of these virus-related pharyngeal cancers consistently exhibit non-keratinised squamous cell carcinoma (SCC) and metastasise to the cervical lymph nodes from a primary small lesion even in the early stage.⁶ On the other hand, virus-related pharyngeal cancers are more sensitive to chemoradiotherapy than non-virus-related pharyngeal cancers,^{7,8} which is associated with a better prognosis in virus-related pharyngeal cancers. Nevertheless, the prognosis of a certain percentage of virus-related NPC cases is poor, with metastasis to the lungs, liver, and bones.⁹ The clinicopathological factors that are correlated with a poor prognosis of virus-related NPC have not yet been clarified.

The global prevalence of type 2 diabetes (T2D) is increasing, and it has become a serious social problem worldwide.¹⁰ T2D has recently been shown to be involved in the development and poor prognosis of various types of malignancies.¹¹ Epigenetic modification, including promoter methylation and microRNA, has been reported as the mechanism of poor prognosis in pancreatic ductal adenocarcinoma and non-B, non-C hepatocellular carcinoma complicated with T2D.^{12–14} T2D causes the deterioration of immune function and immunocompromised states.¹⁵ Interestingly, the incidence of EBV infection has been consistently reported to be high in T2D patients.^{16,17} Epithelial–mesenchymal transition (EMT) is one of the mechanisms by which diabetes promotes

the development of cancer.¹⁸ EMT represents a series of changes in which epithelial cells lose their characteristics, reorganise the cytoskeleton, and obtain the ability to infiltrate and metastasise by acquiring the phenotype of mesenchymal cells.¹⁹ It has recently been reported that EBV infection causes EMT and leads to further malignancy of NPC.²⁰ There is no consensus on HPV infection, which is an important facilitator of SCC. These findings suggest the possibility that the incidence and prognosis of EBV-positive NPC can be influenced by T2D. Herein, we investigated the influence of T2D on virus-related NPC, focusing on prognosis and EMT.

MATERIALS AND METHODS

Cases

NPC cases were obtained from the case database of Hirosaki University Hospital and related facilities. All cases were pathologically diagnosed with NPC with a histological type of SCC in routine practice. Formalin-fixed, paraffin-embedded blocks and tissue slides of all cases from January 2010 to December 2020 were retrieved from hospital files. All specimens were biopsy tissue collected before the patient underwent radiation therapy or chemotherapy. A retrospective study was conducted based on clinicopathological data extracted from medical records. Patients who met the T2D criteria advocated by the Japan Diabetes Foundation were considered diabetic patients.²¹ A total of 70 patients were screened and divided into a diabetic group (DM, 21 males and two females) and a non-diabetic group (nDM, 34 males and 13 females). Regarding the history of drinking alcohol, daily habitual alcohol intake of 40 g or more for men and 20 g or more for women was considered an example of drinking. For hypertension, cases with blood pressure of 140/90 mmHg or higher or a history of treatment for hypertension were defined as hypertensive cases. Dyslipidaemia was defined as a total serum cholesterol level of 5.7 mmol/L or higher, a triglyceride level of 1.7 mmol/L or higher, or a prescription for dyslipidaemia. Regarding smoking habits, smoking cases were defined as those who continued smoking within one year from the start of treatment. Cumulative smoking volume was evaluated by pack year. The number of metastases in the cervical lymph nodes was evaluated using the images of positron emission tomography-computed tomography (PET-CT) images during routine admission.

Histopathological assessment

Histopathological evaluation was performed by two pathologists (HM and KK) using H&E-stained sections of each sample. The pathological diagnosis of NPC was classified into keratinised SCC, non-keratinised SCC, and undifferentiated SCC according to the criteria of the World Health Organization 2017 (WHO).^{22,23} Histological types classified as basaloid SCC were excluded because they were not present within the period of this study.

Immunohistochemistry and *in situ* hybridisation

Immunohistochemistry (IHC) and *in situ* hybridisation (ISH) were performed using an automated immunostainer (Bond autostainer; Leica Biosystems, Germany) as previously described.²⁴ The immunostaining antibodies used were p16 (Clone E6H4, prediluted; Ventana Medical Systems, USA), E-cadherin (Clone NCH-38; Dako, Agilent Technologies, USA), and vimentin (Clone V9; Leica Biosystems). An EBER probe (Bond ISH EBER Probe, CAT # PB0589; Leica Biosystems) was used for EBER ISH, and a HPV probe (Bond HPV Probe, subtypes 16, 18, 31, 33, and 51, CAT # PB0829; Leica Biosystems) was used for high-risk HPV ISH. p16 immunostaining was considered positive when 75% or more of the cell nuclei of the tumour cells showed strong staining.²⁵ Partial staining of less than 75% was considered negative.

The evaluation of E-cadherin and vimentin was performed using the classical pathological method (semiquantitative evaluation) by Tsoukalas *et al.* with some modifications.²⁶ In particular, two parameters were evaluated: the intensity of staining (weak or diffuse, with background staining considered negative) and the percentage (%) of tumour cells stained (both membranous and cytoplasmic for vimentin and membranous alone for E-cadherin). Finally, the percentage of tumour cells that were positive (weakly positive or greater) was calculated.

The determination of EBER-I ISH-positive results was based on the criteria for cases in which strong brown staining was observed in the nucleus.²⁷ The determination of high-risk HPV ISH-positive results was based on the observation of diffuse and punctate positive signals in the nuclei of tumour cells.²⁶

Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Autonomous Medical University, Japan). Disease-specific survival (DSS) was defined as the period from the start of first-line treatment to death from NPC. Overall survival (OS) was defined as the period from the start of first line treatment to death from any cause. Continuous variables were analysed using the non-parametric method (Mann–Whitney U test) for non-normally distributed data and expressed as the median (range). Categorical variables were analysed using the chi-square test or Fisher exact test as appropriate and are expressed as a number (%). Variables with a *p* value less than 0.05 in the univariate analysis were considered candidates for multivariate analysis using the Cox proportional hazard model. Survival curves were constructed using Kaplan–Meier analysis, and *p* values were determined by the log-rank test for censored survival data. A *p* value <0.05 was considered statistically significant.

RESULTS

Clinicopathological features of NPC complicated with T2D

The clinicopathological features of the study patients are summarised in [Supplementary Table 1 \(Appendix A\)](#). Although the mean age tended to be higher in DM patients (69.0±10.9 years) than in nDM patients (60.1±14.7 years), the difference was not significant. Body mass index was similar between nDM patients (21.5±3.4 kg/m²) and DM patients (21.9±3.1 kg/m²). Pretreatment glycated haemoglobin (HbA1c) and fasting blood glucose levels were significantly higher in DM patients than in nDM patients (*p*<0.01). The prevalence of dyslipidaemia and hypertension was significantly higher in DM patients than in nDM patients (*p*<0.05), while there were no significant differences in the prevalence of smoking or drinking habits. Tumour size in DM patients tended to be larger than that in nDM subjects. Tumour stage, nodal stage, metastasis stage, AJCC stage, and the prevalence of recurrence were comparable between the two groups. In the pathological assessment, the prevalence of keratinised SCC in nDM patients was significantly higher than in DM patients (23.4% vs 8.7%, *p*<0.01). Conversely, the prevalence of undifferentiated carcinoma in DM patients was significantly higher than in nDM patients (78.2% vs 55.3%, *p*<0.01).

Pathological findings and virus infection status in NPC

Tumour cells of keratinised SCC in the nDM patients showed relatively abundant cytoplasm with the production of keratinised materials ([Fig. 1A](#)). EBER-I expression was absent on ISH ([Fig. 1B](#)). In contrast, tumour cells of non-keratinised SCC in DM patients showed narrow cytoplasm and dense nuclear chromatin without the production of keratinised materials ([Fig. 1C](#)). EBER-I ISH showed strong nuclear expression ([Fig. 1D](#)). Some keratinised SCCs ([Fig. 1E,F](#)) and non-keratinised SCCs ([Fig. 1G,H](#)) showed a faint positive reaction for p16 in the nucleus ([Fig. 1E,G](#)) and a negative reaction for HPV-DNA ISH ([Fig. 1F,H](#)). In the nDM patients, the frequency of EBER-positive cases was 68.1% (32/47 patients), while 91.3% of patients (21/23 patients) were EBER-positive in the DM patients (*p*<0.01) ([Fig. 1I](#)). Among all patients, 7.1% (5/70 patients) were both p16-positive and high-risk

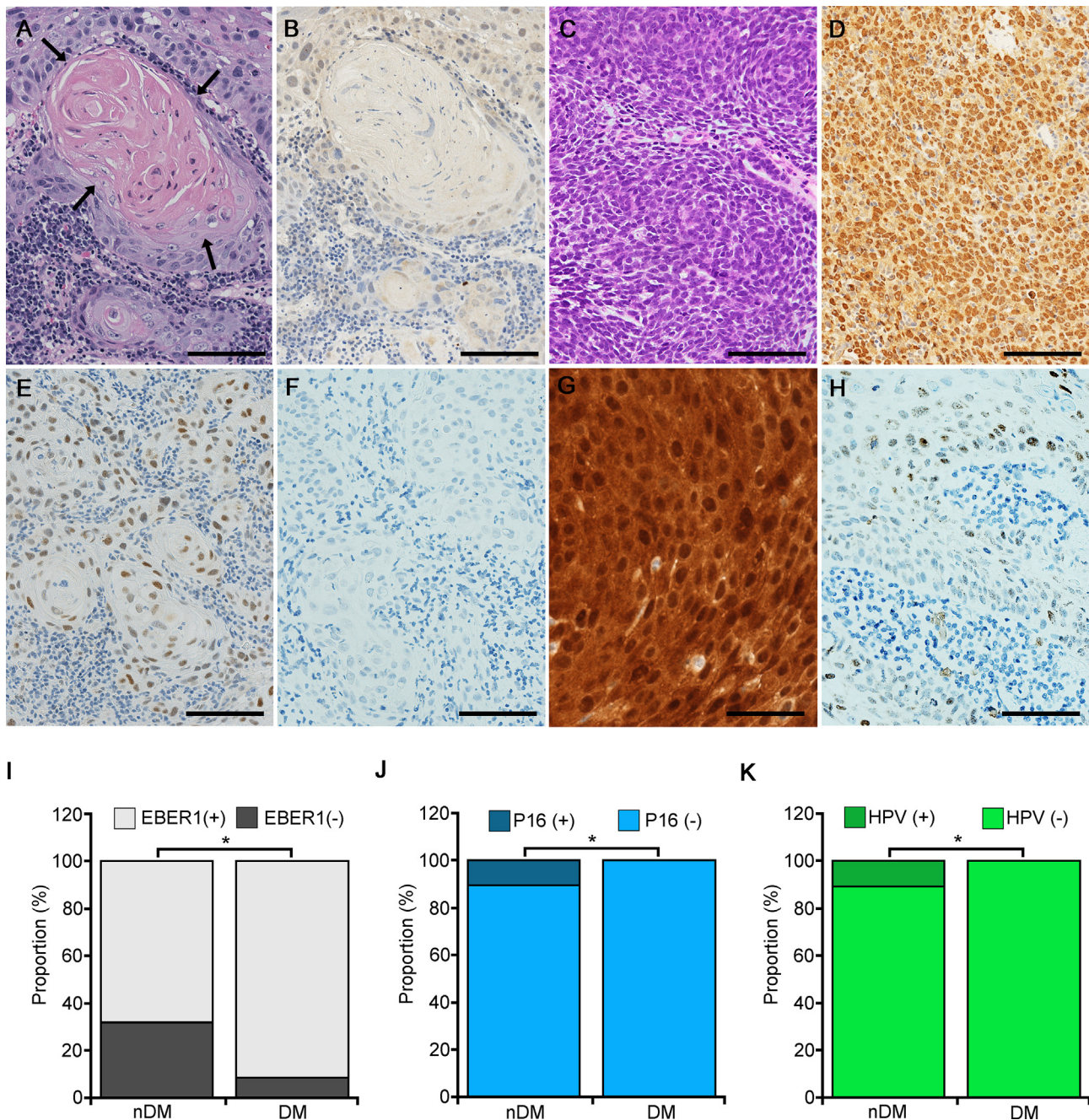


Fig. 1 Histological evaluation of NPC infected with EBV and HPV. Tumour cells showed intercellular bridges and keratinisation (arrow) in nasopharyngeal keratinised SCC in the nDM patients (A). In the same part in Panel A, ISH for EBER-I showed a lack of expression (B). Tumour cells displayed a high N:C ratio without brisk keratinisation in non-keratinising undifferentiated carcinoma in the DM patients (C). In Panel C, ISH showed diffusely positive nuclei in cancer cells for EBER-I (D). IHC for p16 (E) was sporadically positive, while ISH for high-risk HPV (F) was negative for nuclei in the tumour cells in some keratinised SCC cases. In non-keratinising undifferentiated SCC, a few cases showed diffuse positivity for p16 IHC in the nuclei and cytoplasm of tumour cells (G), while ISH for high-risk HPV showed a punctate appearance in the nucleus of tumour cells (H). The proportion of EBER-I ISH-positive cases of DM was significantly higher than that of nDM (I). The proportion of p16 IHC-positive cases (J) and high-risk HPV ISH-positive cases (K) was comparable between nDM and DM patients. DM, diabetic group; EBV, Epstein–Barr virus; HPV, human papillomavirus; IHC, immunohistochemistry; ISH, in situ hybridisation; nDM, non-diabetic group; NPC, nasopharyngeal carcinoma; SCC, squamous cell carcinoma. p values <0.05 were considered significant; * p <0.01. Bars represent 50 μ m.

HPV-DNA ISH-positive regardless of keratinisation, and all of those patients were nDM patients (p <0.01) (Fig. 1J,K).

Impacts of T2D on EMT progression in NPC

To investigate the association between T2D and EMT in NPC, IHC of E-cadherin and vimentin was performed. E-cadherin was circumferentially expressed on the cell

membrane of nasopharyngeal SCC tumour cells in the nDM patients (Fig. 2A). In contrast, there was an increase in the prevalence of patients with decreased expression of E-cadherin in the DM patients (Fig. 2B). Semiquantitative evaluation of E-cadherin expression showed that EBER-I expression had no impact on E-cadherin expression in the nDM patients, while E-cadherin expression was significantly reduced in the DM patients compared to the nDM patients

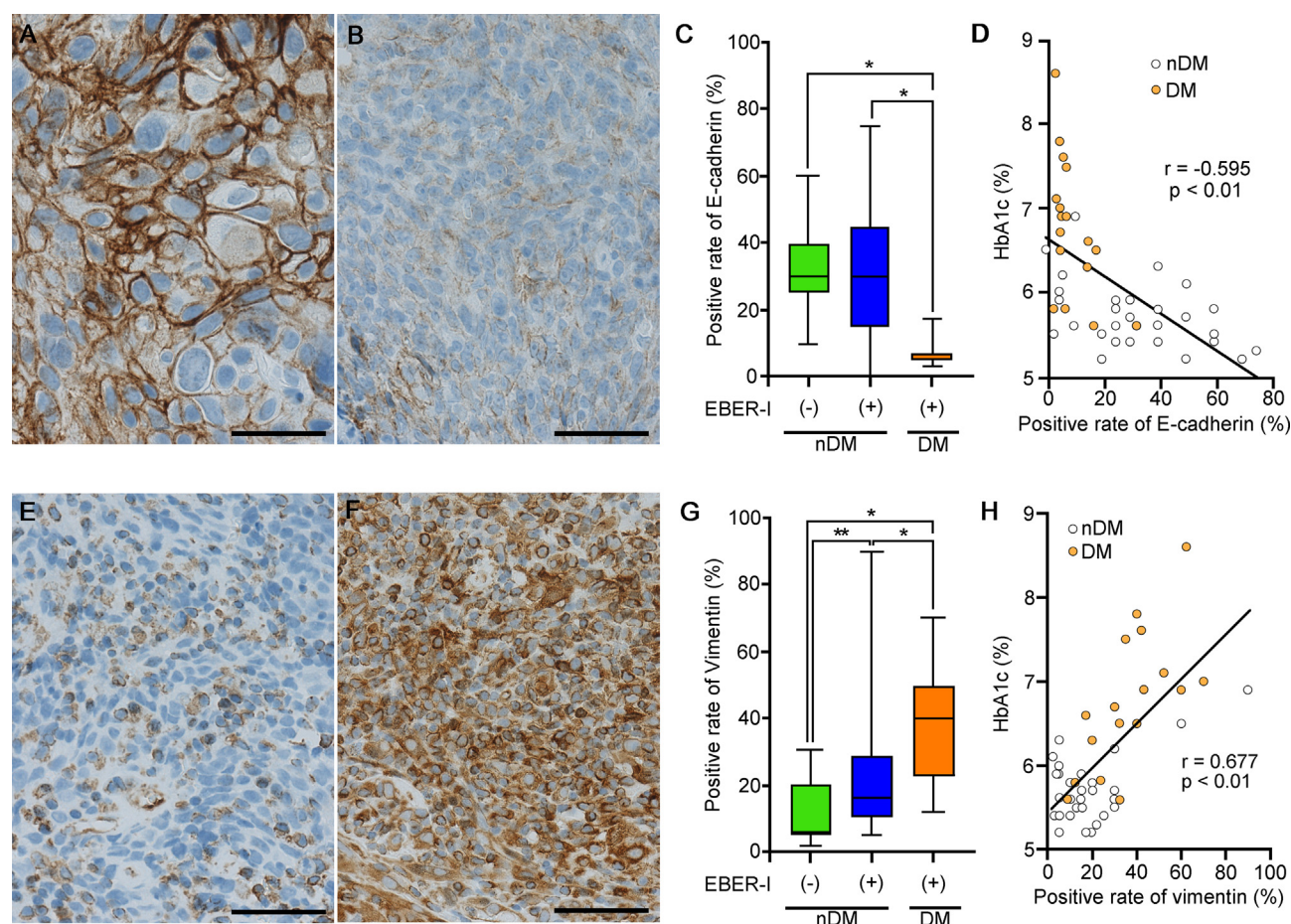


Fig. 2 Influence of diabetes on the expression of EMT markers. E-cadherin expression in IHC was retained in the cell membrane in nDM patients (A) and reduced in DM group (B). EBV infection had no impact on E-cadherin expression in nDM patients, while the expression was significantly decreased in DM patients, restricted to EBV-positive patients (C). The level of HbA1c was negatively correlated with the positive rate of E-cadherin in NPC patients (D). Vimentin expression in IHC was minimal in the cytoplasm of tumour cells in nDM patients (E). Vimentin expression was significantly increased in DM patients (F). Vimentin expression was significantly increased in EBV-positive nDM patients compared to EBV-negative nDM patients (G). Vimentin expression was further increased in DM patients compared to nDM patients, restricted to EBV-positive patients. The level of HbA1c was proportionally correlated with the positive rate of vimentin in NPC patients (H). DM, diabetic group; EBV, Epstein–Barr virus; EMT, epithelial–mesenchymal transition; IHC, immunohistochemistry; nDM, non-diabetic group; NPC, nasopharyngeal carcinoma. The data are presented as the mean \pm SD. Statistical analysis was performed by two-way ANOVA with post hoc multiple comparison tests for C,G and by Pearson's correlation analysis for D,H. p values <0.05 were considered significant; * $p<0.01$ and ** $p<0.05$. Bars represent 50 μ m.

regardless of EBER-I positivity ($p<0.01$) (Fig. 2C). The positive rate of E-cadherin was negatively correlated with the HbA1c value ($r = -0.60$, $p<0.01$) (Fig. 2D). On the other hand, vimentin expression was scarcely observed in the cytoplasm of tumour cells in the nDM patients (Fig. 2E), while vimentin expression was increased in the DM patients (Fig. 2F). Semiquantitative evaluation showed that vimentin expression in EBER-I-positive nDM patients was significantly increased compared to that in EBER-I-negative nDM patients ($p<0.05$) (Fig. 2G). Furthermore, vimentin expression was significantly increased in EBER-I-positive DM patients than EBER-I-positive nDM patients ($p<0.01$). In contrast to the expression of E-cadherin, vimentin expression was proportionally correlated with the HbA1c value ($r = 0.68$, $p<0.01$) (Fig. 2H).

Determination of the optimal cut-off value of HbA1c and fasting blood glucose by ROC analysis

Because the HbA1c value was correlated with both E-cadherin and vimentin expression in NPC, receiver operating characteristic (ROC) analysis was performed to

explore the performance of HbA1c and fasting blood glucose (FBG) as prognostic predictors to determine their cut-off value. The area under the curve (AUC) of the FBG level was 0.63 and 0.62 for death at 2 and 3 years after the start of treatment, respectively (Fig. 3A,B). The sensitivity and specificity of the test at a cut-off value of 6.4 mmol/L in cases of primary death at 2 years were 0.60 and 0.67, respectively. On the other hand, the AUCs of HbA1c were 0.76 and 0.72 for death at 2 and 3 years after the start of treatment, respectively (Fig. 3C,D). The sensitivity and specificity of the test at a cut-off value of 6.5% in cases of primary death at 2 years were 0.64 and 0.81, respectively, indicating that HbA1c was superior to fasting blood glucose in prognostic ability.

Correlation between the number of metastasised cervical lymph nodes detected on PET-CT and HbA1c level

To investigate the effects of glycaemic control on the frequency of cervical lymph node metastasis, we examined the relationship between HbA1c and the number of cervical

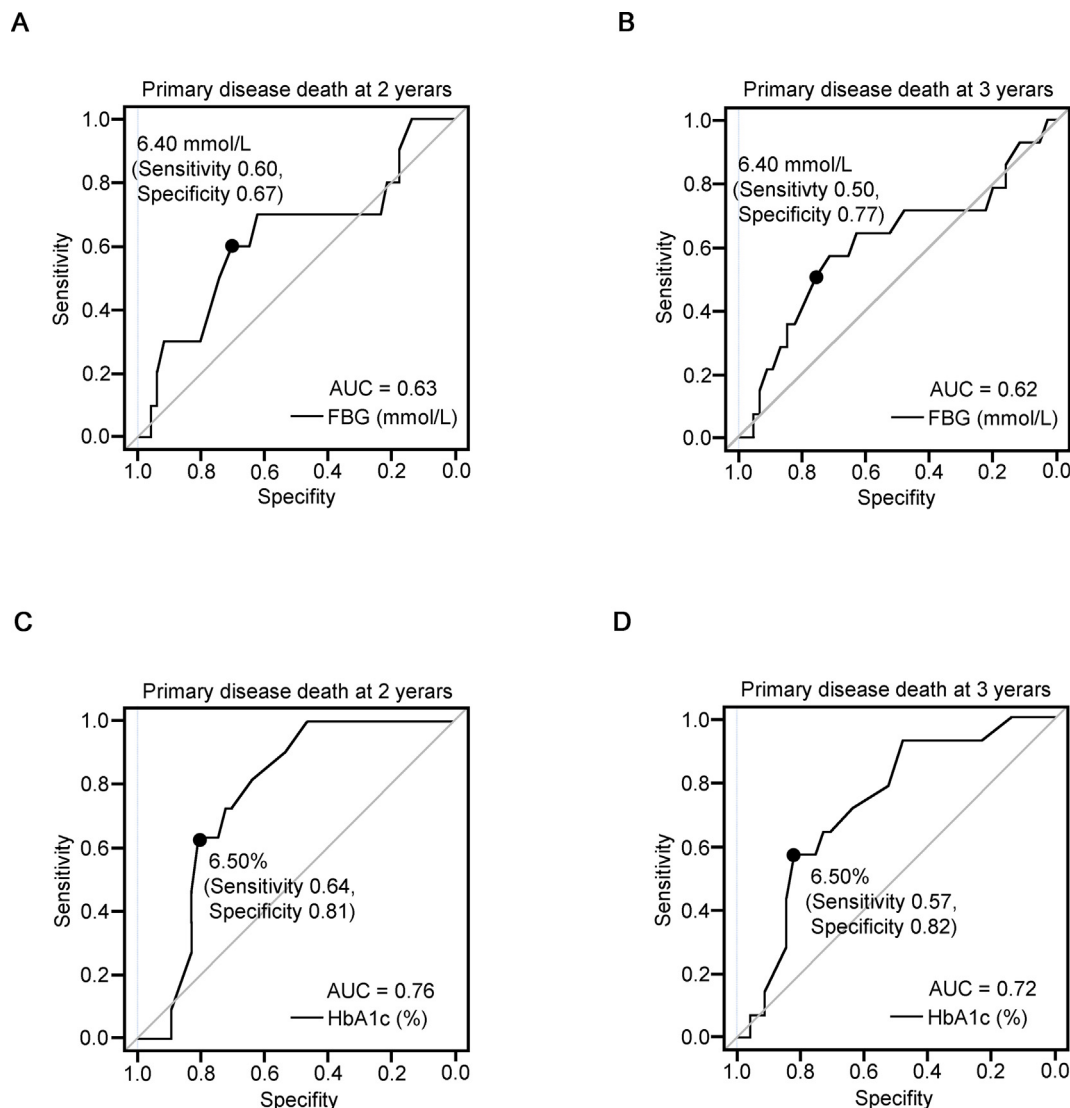


Fig. 3 ROC analysis for optimal cut-off values of HbA1c and fasting blood glucose. The ROC curve between FBG and primary disease death at 2 years had an AUC of 0.63 (A). The sensitivity and specificity of the test at a cut-off value of 6.40 mmol/L were 0.60 and 0.67, respectively. The ROC curve between FBG and primary disease death at 3 years had an AUC of 0.62 (B). The sensitivity and specificity of the test at a cut-off value of 6.4 mmol/L were 0.50 and 0.77, respectively. The ROC curve between HbA1c and primary disease death at 2 years had an AUC of 0.76 (C). The sensitivity and specificity of the test at a cut-off value of 6.50% were 0.64 and 0.81, respectively. The ROC curve between HbA1c and primary disease death at 3 years had an AUC of 0.72 (D). The sensitivity and specificity of the test at a cut-off value of 6.50% were 0.57 and 0.82, respectively. AUC, area under the curve; FBG, fasting blood glucose.

lymph node metastases in patients undergoing PET-CT imaging. PET-CT imaging visualised a single metastatic lymph node as red in the coronal and axial directions in the nDM patients (Fig. 4A), while the multiple positive signals were identified in the DM patients (Fig. 4B). The number of cervical lymph node metastases was significantly increased in patients with HbA1c $\geq 6.5\%$, the cut-off determined by ROC analysis, compared to those with HbA1c $< 6.5\%$ (5.30 vs 2.75, $p < 0.01$) (Fig. 4C). HbA1c levels and the number of cervical lymph node metastases were positively correlated ($r = 0.21$, $p < 0.01$) (Fig. 4D). The correlation between EMT properties and the number of lymph node metastases was examined. The frequency of E-cadherin-positive cells showed a significant negative correlation with the number of lymph node metastases ($r = -0.30$, $p < 0.05$) (Fig. 4E), while there was a significant positive correlation between the frequency of vimentin-positive cells and the number of cervical lymph node metastases ($r = 0.39$, $p < 0.01$) (Fig. 4F).

Survival curves of patients with NPC complicated with T2D

The Kaplan–Meier survival curve showed that DSS was significantly shorter in the DM patients than in the nDM patients (Fig. 5A). DSS was also significantly shorter in cases with HbA1c values $\geq 6.5\%$ than in cases with HbA1c values $< 6.5\%$ (Fig. 5B). The Kaplan–Meier survival curve by EBER-I status showed that patients with EBER-I-positive NPC tended to have longer DSS than those with EBER-I-negative NPC, but the difference was not statistically significant (Fig. 5C). However, patients with EBER-I-positive NPC complicated with T2D had a much worse prognosis than non-diabetic NPC patients positive for EBER-I ($p < 0.01$) (Fig. 5D). Patients with EBER-I-positive NPC complicated with T2D had a similar prognosis to non-diabetic patients with EBER-I-negative NPC ($p = 0.11$). The Kaplan–Meier survival curve also showed that OS was

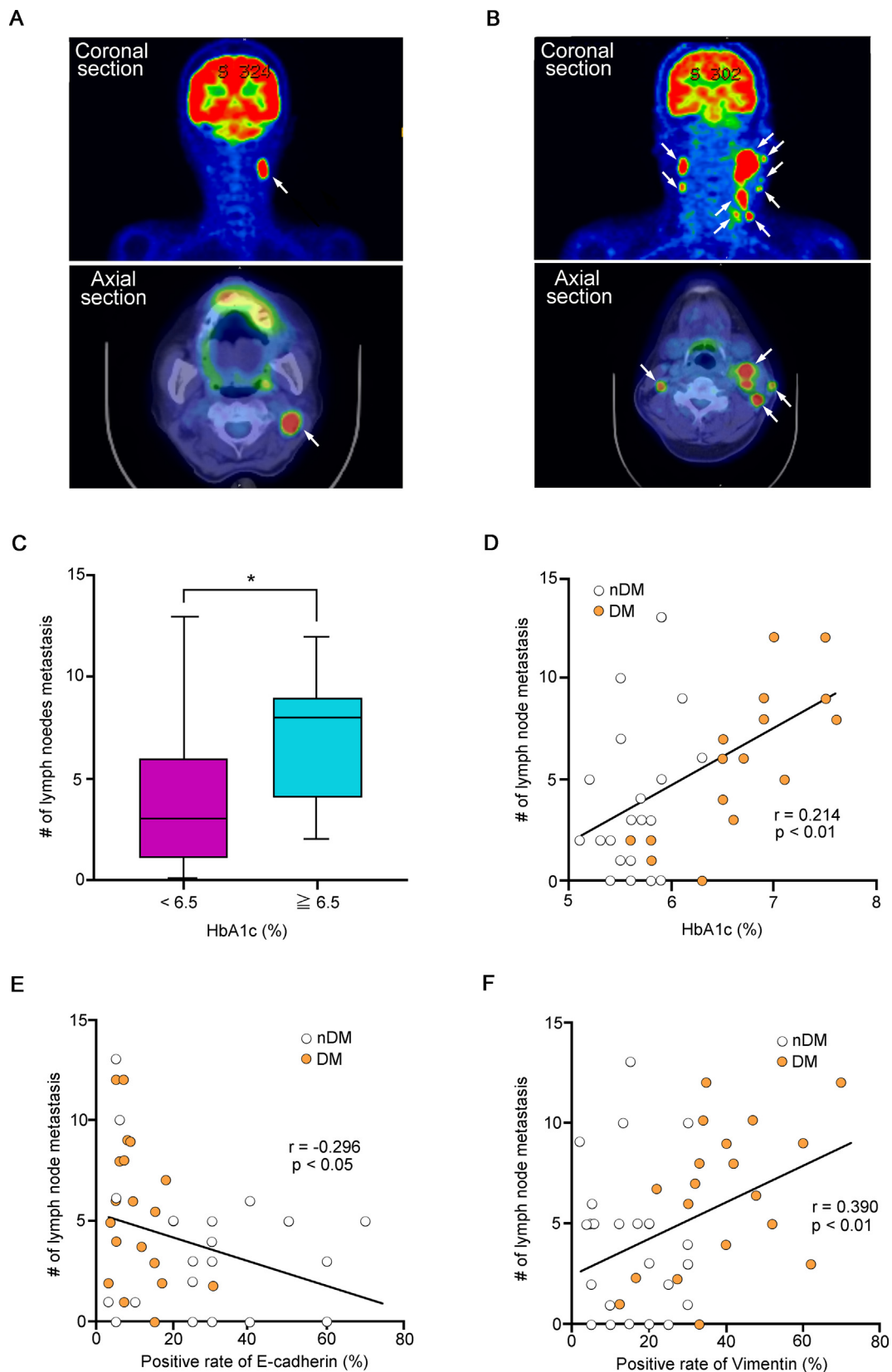


Fig. 4 Correlation between the number of PET-CT-positive metastatic cervical lymph nodes and HbA1c. PET-CT imaging visualised a single metastatic cervical lymph node (arrow) from the coronal and axial directions in nDM patients (A) and multiple metastatic lymph nodes (arrows) in DM patients (B). The number of lymph node metastases in patients with HbA1c $\geq 6.5\%$ was significantly higher than that in patients with HbA1c $< 6.5\%$ (C). The number of lymph node metastases in NPC patients was proportionally correlated with HbA1c level (D). The number of lymph node metastases in NPC patients was negatively correlated with the positive rate of E-cadherin (E). The number of lymph node metastases in NPC patients was proportionally correlated with the positive rate of vimentin (F). DM, diabetic group; nDM, non-diabetic group. The data are presented as the mean \pm SD. Statistical analysis was performed by two-way ANOVA with post hoc multiple comparison tests for C and by Pearson's correlation analysis for D–F. p values < 0.05 were considered significant; $*p < 0.01$.

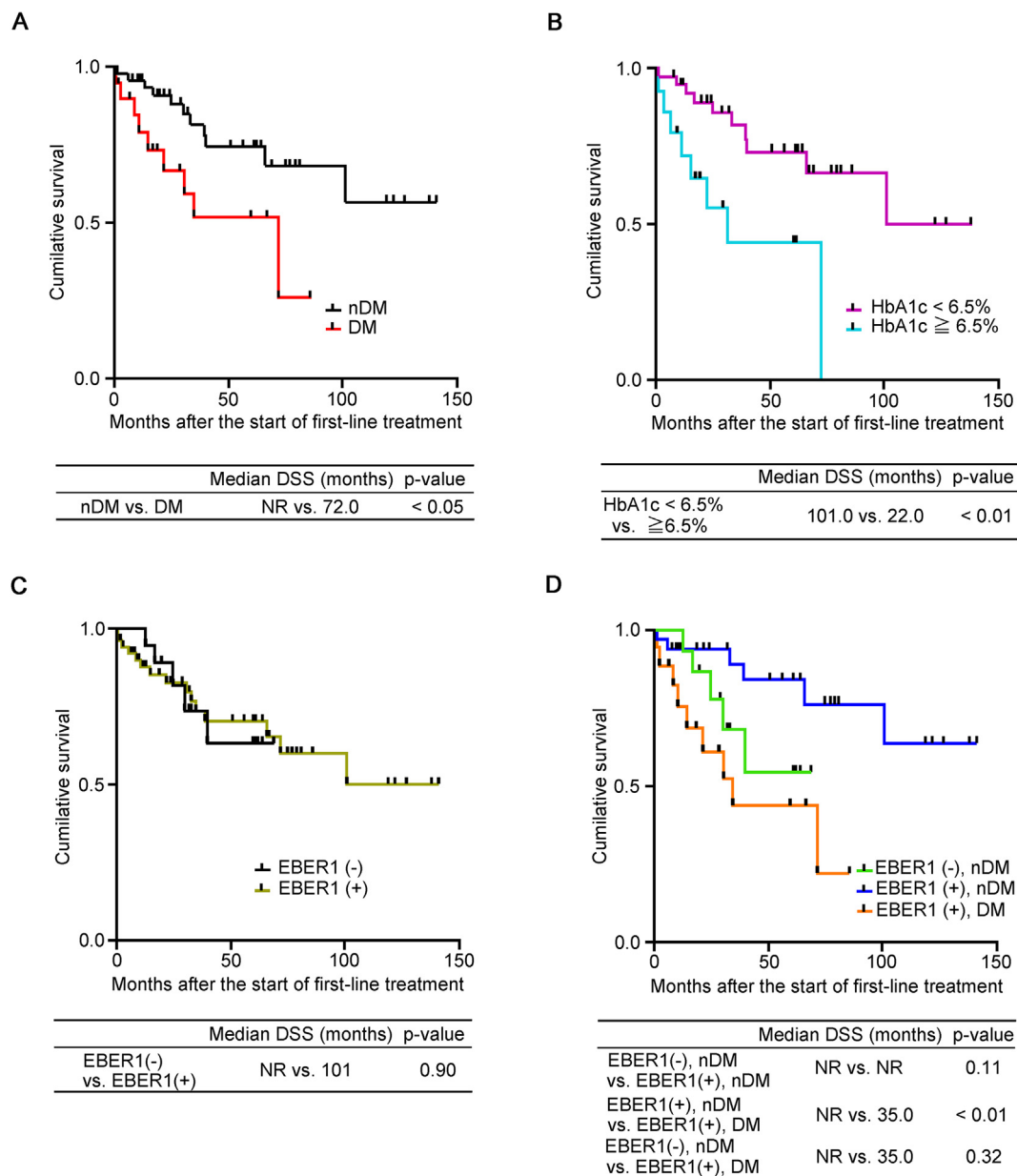


Fig. 5 Kaplan–Meier analysis comparing disease-specific survival in nasopharyngeal squamous cell carcinoma. Kaplan–Meier survival curves showed a shortened DSS in DM patients compared to nDM patients (A). DSS was shortened in patients with HbA1c $\geq 6.5\%$ compared to those with HbA1c $< 6.5\%$. (B). EBER-I positivity had no impact on DSS in NPC patients (C). Patients with EBER-I-positive NPC complicated with diabetes had a significantly worse prognosis than non-diabetic patients with EBER-I-positive NPC ($p < 0.01$) (D). The DSS of patients with EBER-I-positive NPC complicated with diabetes was similar to that of non-diabetic patients with EBER-I-negative NPC. DM, diabetic group; DSS, disease-specific survival; nDM, non-diabetic group; NPC, nasopharyngeal carcinoma; NR, not reached.

significantly shorter in DM patients than in nDM patients (Supplementary Fig. 1A, Appendix A) and in the patients with HbA1c values $\geq 6.5\%$ than in the patients with HbA1c values $< 6.5\%$ (Supplementary Fig. 1B, Appendix A). EBER-I status had no significant impact on OS in NPC patients (Supplementary Fig. 1C, Appendix A). Patients with EBER-I-positive NPC complicated with T2D had a much worse OS than non-diabetic NPC patients positive for EBER-I ($p < 0.01$) (Supplementary Fig. 1D, Appendix A).

In a univariate analysis of DSS, history of T2D ($p < 0.01$), duration of T2D ≥ 5.0 years ($p < 0.05$), HbA1c levels $\geq 6.5\%$, and smoking habits ($p < 0.01$) were significant risk factors for decreased survival (Table 1). A history of metabolic disorders other than T2D, such as hypertension and dyslipidaemia, did not correlate with DSS. In addition, the status of

P16 expression in immunohistochemistry and EBER-I ISH did not correlate with DSS. Multivariate analysis further confirmed that HbA1c levels $\geq 6.5\%$ ($p < 0.05$) and smoking habits ($p < 0.05$) remained significant risk factors for decreased survival (Table 2). Univariate analysis of OS also showed that history of diabetes mellitus ($p < 0.01$), duration of diabetes mellitus ≥ 5 years ($p < 0.01$), HbA1c levels $\geq 6.5\%$ ($p < 0.01$), FBG levels ≥ 7.0 mmol/L ($p < 0.05$), and smoking habits ($p < 0.05$) were significant risk factors for decreased survival (Supplementary Table 2 Appendix A). Multivariate analysis further confirmed that HbA1c values $\geq 6.5\%$ ($p < 0.05$) and smoking habits ($p < 0.05$) remained significant risk factors for decreased survival after adjustment for other correlated clinical factors (Supplementary Table 3, Appendix A).

Table 1 Univariate analysis (disease-specific survival)

	Median DSS (months)	<i>p</i> value
Age (years): <65.0 vs ≥65.0	101 vs 72	0.88
Male vs Female	101 vs not reached	0.18
BMI (kg/m ²): <25.0 vs ≥25.0	101 vs not reached	0.36
Tumour size (mm): <28.0 vs ≥28.0	Not reached vs 72	0.11
Histological differentiation (keratinising vs non-keratinising and undifferentiated)	Not reached vs not reached	0.94
T1-2 vs T3-4 (AJCC)	Not reached vs 72	0.28
N: (–) vs (+)	Not reached vs 101	0.73
Alcohol habit: (–) vs (+)	Not reached vs 101	0.16
History of T2D: (–) vs (+)	Not reached vs 35	<0.01
Duration of T2D (years): <5.0 vs ≥5.0	Not reached vs 31	<0.05
HbA1c (%): <6.5 vs ≥6.5	Not reached vs 22	<0.01
Fasting blood glucose (mmol/L): <6.6 vs ≥6.6	Not reached vs not reached	0.37
History of hypertension: (–) vs (+)	Not reached vs 72	0.35
History of dyslipidaemia: (–) vs (+)	Not reached vs 52	0.10
Smoking habits (overall): (–) vs (+)	Not reached vs 65	<0.01
p16 expression in immunohistochemistry: (–) vs (+)	101 vs not reached	0.67
EBER-I in situ hybridisation: (–) vs (+)	Not reached vs not reached	0.49

DSS, disease-specific survival; BMI, body mass index.

Table 2 Multivariable analysis for the Cox proportional hazard regression model for disease-specific survival

	Hazard ratio	95% CI	<i>p</i> value
History of T2D: (–) vs (+)	0.30	0.04–2.20	0.23
Duration of T2D (years): <5.0 vs ≥5.0	3.41	0.82–14.3	0.09
HbA1c (%): ≥6.5	6.84	1.27–36.8	<0.05
Smoking habits (overall): (–) vs (+)	5.15	1.39–18.8	<0.05

CI, confidence interval; T2D, type 2 diabetes.

DISCUSSION

In this study, we first clarified that the proportion of EBER-I-positive NPC was significantly higher in the DM patients than in the nDM patients. Patients with nasopharyngeal SCC complicated with T2D showed a significantly higher prevalence of EMT than patients without T2D. This change was significantly correlated with the level of HbA1c. An HbA1c value of 6.5% was determined as a cut-off value for primary disease death at 2 years, which corresponded to the diagnostic criteria for T2D by the Japan Diabetes Foundation.¹⁹ The presence of T2D exacerbated DSS and OS in the patients with nasopharyngeal SCC in this study, particularly in the EBER-I-positive patients. Furthermore, the number of lymph node metastases was increased in the patients with HbA1c value >6.5%. HbA1c value >6.5% remained a significant risk factor for NPC in multivariate analysis. Thus, diabetes may be regarded as a risk factor for developing nasopharyngeal SCC, particularly in patients with HbA1c value >6.5%.

One meta-analysis reported that diabetes decreased NPC risk and had no impact on prognosis.²⁸ However, detailed clinical information on diabetes was lacking in that meta-analysis, which was based solely on the presence of diabetes and blood glucose levels. In contrast, the detailed

diabetes states evaluated in this study were based on accurate records of diabetes, including HbA1c at NGSP, FBG, and duration and treatment of diabetes. Considering these data, we showed that a high HbA1c level significantly worsened the status of lymph node metastasis and the DSS and OS of nasopharyngeal SCC, particularly in the patients related to EBV. These detailed assessments also enabled us to determine that the cut-off value of HbA1c as a marker for poor prognosis is 6.5%.

In Japan, unlike other Asian regions, there were a certain number of non-EBV-related NPC patients.²⁹ This enabled us to directly compare EBV-dependent nasopharyngeal SCC to EBV-independent nasopharyngeal SCC in this study. Our results suggested that EBV-related nasopharyngeal SCC was strongly associated with T2D. In general, infectious diseases are more frequent and/or severe in patients with DM, which potentially increases their mortality.³⁰ The greater frequency of infections in T2D patients is caused by the hyperglycaemic environment that favours immune dysfunction (e.g., damage to neutrophil function, depression of the antioxidant system).³¹ Therefore, our result is reasonable considering the immunocompromised nature of nasopharyngeal SCC patients complicated with T2D.

Although virus-related pharyngeal SCC is assumed to have a better prognosis because of its good response to chemoradiotherapy,^{7,8} the prognosis of nasopharyngeal SCC was unrelated to the presence of EBV infection in this study. Although the precise reason for this was unclear, the relative abundance of diabetic patients (23/70 patients) would worsen the prognosis of EBV-related NPCs in our study. Furthermore, EBV infection had no impact on prognosis in NPC patients without diabetes. Because this may be ascribed to the small sample number, a large-scale study will be needed in the future.

Keratinising SCC was evident in nDM patients in the present study. This may be ascribed to the higher rate of EBV infection in DM patients because EBV infection is strongly associated with the development of non-keratinising SCC.^{5,6} Conversely, environmental factors other than EBV infection may be more involved in the development of keratinising SCC in nDM patients. Smoking and high-salt diets are known to be risk factors for NPC.^{3,32} Salt intake and smoking rates are high in the areas where this study was conducted. High salt administration has been found to cause keratinising SCC in animal studies.^{33,34} These mechanisms may be involved in the high prevalence of keratinising SCC in nDM patients in this study.

We showed that EMT was accelerated in nasopharyngeal SCC complicated with T2D. Several reports have shown that diabetes is a possible enhancer of EMT.^{12,13,18,35} Our previous study also showed that diabetes accelerates promoter methylation of the *CDH1* gene and expression of miR-105-p, resulting in decrease in the expression of E-cadherin in pancreatic ductal carcinoma patients.^{12,13} Furthermore, EMT is exacerbated in the onset and progression of virus-related cancers, including EBV-related NPC.²⁰ This evidence implies that EMT is promoted by multiple mechanisms in EBV-related nasopharyngeal SCC complicated by T2D, which may worsen the prognosis.

In our study, an HbA1c cut-off value of 6.5% was determined by ROC curve analysis for the treatment of T2D in nasopharyngeal SCC. The hazard ratio of cases with an HbA1c level of 6.5% or more in multivariate analysis for

DSS using the Cox proportional hazard model was 6.84, which was considered to be very high. To suppress the onset and progression of nasopharyngeal SCC, it is important to keep the HbA1c level at <6.5%. In addition to glycaemic control, metformin is known to have antitumoural effects in NPC.³⁶ In this study, none of the antidiabetic agents showed antitumoural effects in NPC, probably due to an insufficient number of patients. It is necessary to re-evaluate with a larger number of nasopharyngeal SCC patients in the future.

Kanno *et al.* reported that smoking is involved in the development of NPC in Japan.²⁹ In this study, 62.9% (44/70 cases) of patients were smokers, suggesting a relationship with smoking, as in previous studies.³⁷ Multivariate analysis also showed that smoking history for the last year was an independent factor of poor prognosis, as was T2D. These findings suggest that T2D and smoking habits can synergistically increase the risk of onset and progression of nasopharyngeal SCC. Intervention and improvement in daily life, including inhibition of smoking, are important for prevention of the onset and development of nasopharyngeal SCC.

In previous studies, SCC and malignant tumours other than SCC were evaluated together as NPCs.³⁸ In contrast, only nasopharyngeal malignant tumours diagnosed pathologically as SCC were evaluated in our study, which could eliminate possible biases resulting from the inclusion of different histological types. Furthermore, performing EBER-I ISH, p16 IHC, and HPV ISH at the same time in all cases enabled the differentiation of non-keratinised SCC. Although five cases of HPV-related nasopharyngeal SCC were observed in this study, no association with T2D was observed. These findings may be ascribed to the difference in EBV infection status, because previous studies show that HPV-positive NPC and EBV-positive NPC seem to be mutually exclusive diseases.^{39,40} The high prevalence of EBV infection in the subjects with diabetic NPC may suppress HPV infection. In uterine cervical carcinoma, which is strongly associated with HPV infection in carcinogenesis, the relationship between diabetes and HPV is still controversial.⁴¹ These results suggest that HPV infection may not be as strongly associated with nasopharyngeal SCC tumourigenesis evoked by diabetes as EBV infection.

In NPC, HPV infection is known to be more likely to occur in Caucasians and is implicated in the pathogenesis of the disease.^{42,43} However, the impact of HPV infection on NPC is inconclusive. Stenmark *et al.* reported that HPV-positive NPC was associated with worse overall survival, progression-free survival, and locoregional control,⁴⁴ while a larger study conducted by Huang *et al.* found that HPV-positive NPC had a greater local symptom burden and larger primary tumours but had similar outcomes compared with EBV-positive NPC subjects.⁴⁰ In Japanese subjects, p16-positive NPC patients represent a minor but not insubstantial proportion of NPC patients, and the proportion of keratinising NPC is small with a larger circumferential tumour extent and no difference in prognosis.⁴⁵ Similarly, prognosis or clinicopathological parameters, including histological type, were comparable between the two groups in the present study. Considering a previous report, it is possible that HPV infection does not have a significant impact on the prognosis of NPC even in non-endemic Japanese patients.

A limitation of this study is that the research was performed mainly in a limited area in the northeastern part of

Japan. As mentioned above, studies with larger cohorts are needed to eliminate regional differences and obtain a sufficient number of nasopharyngeal SCC patients among several institutes. Because most of the histopathological specimens used in this study were biopsy specimens, the size of the specimens was too small to analyse the detailed histological and genetic changes associated with diabetes.

CONCLUSION

T2D is a risk factor for exacerbating the prognosis of nasopharyngeal SCC, particularly EBV-positive SCC, via EMT. Appropriate glycaemic control (HbA1c <6.5%), along with intervention in life customs such as smoking, is an important determinant of the prognosis of nasopharyngeal SCC complicated with T2D.

Ethics approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Hirosaki University Graduate School of Medicine (approval number #2021–054).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.pathol.2023.09.013>.

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References

- Chen YP, Chan ATC, Le QT, *et al.* Nasopharyngeal carcinoma. *Lancet* 2019; 394: 64–80.
- Carioli G, Negri E, Kawakita D, *et al.* Global trends in nasopharyngeal cancer mortality since 1970 and predictions for 2020: focus on low-risk areas. *Int J Cancer* 2017; 140: 2256–64.
- Seino T, Saito Y. *The National Health and Nutrition Survey in Japan*. Ministry of Health, Labour and Welfare; 2022, cited 26 Jul 2022. https://www.mhlw.go.jp/stf/newpage_14156.html
- Gillison ML, Koch WM, Capone RB, *et al.* Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000; 92: 709–20.
- Tsao SW, Tsang CM, Lo KW. Epstein-Barr virus infection and nasopharyngeal carcinoma. *Philos Trans R Soc Lond B Biol Sci* 2017; 372: 20160270.
- Wang S, Claret FX, Wu W. MicroRNAs as therapeutic targets in nasopharyngeal carcinoma. *Front Oncol* 2019; 9: 756.
- Tan WL, Tan EH, Lim DW, *et al.* Advances in systemic treatment for nasopharyngeal carcinoma. *Chin Clin Oncol* 2016; 5: 21.
- Berman TA, Schiller JT. Human papillomavirus in cervical cancer and oropharyngeal cancer: one cause, two diseases. *Cancer* 2017; 123: 2219–29.
- Chiang AK, Mak NK, Ng WT. Translational research in nasopharyngeal carcinoma. *Oral Oncol* 2014; 50: 345–52.
- Zhou B, Lu Y, Hajifathalian K, *et al.* Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants. *Lancet* 2016; 387: 1513–30.

11. Srivastava SP, Goodwin JE. Cancer biology and prevention in diabetes. *Cells* 2020; 9: 1380.
12. Saito T, Mizukami H, Umetsu S, *et al.* Worsened outcome in patients with pancreatic ductal carcinoma on long-term diabetes: association with E-cadherin 1 (CDH1) promoter methylation. *Sci Rep* 2017; 7: 18056.
13. Hara Y, Mizukami H, Yamazaki K, *et al.* Dual epigenetic changes in diabetes mellitus-associated pancreatic ductal adenocarcinoma correlate with downregulation of E-cadherin and worsened prognosis. *J Pathol Clin Res* 2023; 9: 354–66.
14. Umetsu S, Mizukami H, Saito T, *et al.* Diabetes, an independent poor prognostic factor of non-B non-C hepatocellular carcinoma, correlates with dihydropyrimidinase-like 3 promoter methylation. *Sci Rep* 2020; 10: 1156.
15. Moutschen MP, Scheen AJ, Lefebvre PJ. Impaired immune responses in diabetes mellitus: analysis of the factors and mechanisms involved. Relevance to the increased susceptibility of diabetic patients to specific infections. *Diabete Metab* 1992; 18: 187–201.
16. Dworżański J, Drop B, Kliszczewska E, Strycharz-Dudziak M, Polz-Dacewicz M. Prevalence of Epstein-Barr virus, human papillomavirus, cytomegalovirus and herpes simplex virus type 1 in patients with diabetes mellitus type 2 in south-eastern Poland. *PLoS One* 2019; 14: e0222607.
17. Mahendra J, Mahendra L, Divya D, *et al.* Association of Epstein-Barr virus, cytomegalovirus and lipocalin with periodontitis in type 2 diabetic subjects. *Oral Dis* 2023; 29: 1163–71.
18. Viedma-Rodríguez R, Martínez-Hernández MG, Martínez-Torres DI, *et al.* Epithelial mesenchymal transition and progression of breast cancer promoted by diabetes mellitus in mice are associated with increased expression of glycolytic and proteolytic enzymes. *Horm Cancer* 2020; 11: 170–81.
19. Thierry JP, Acloque H, Huang RY, *et al.* Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; 139: 871–90.
20. Chen X, Bode AM, Dong Z, *et al.* The epithelial-mesenchymal transition (EMT) is regulated by oncoviruses in cancer. *FASEB J* 2016; 30: 3001–10.
21. Seino Y, Nanjo K, Tajima N, *et al.* Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *J Diabetes Investig* 2010; 1: 212–28.
22. Stelow EB, Wenig BM. Update from the 4th Edition of the World Health Organization classification of head and neck tumours: nasopharynx. *Head Neck Pathol* 2017; 11: 16–22.
23. Lydiatt WM, Patel SG, O'Sullivan B, *et al.* Head and neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA Cancer J Clin* 2017; 67: 122–37.
24. Uchida C, Mizukami H, Hara Y, *et al.* Diabetes in humans activates pancreatic stellate cells via RAGE in pancreatic ductal adenocarcinoma. *Int J Mol Sci* 2021; 22: 11716.
25. Chi J, Preeshagul IR, Sheikh-Fayyaz S, *et al.* Evaluating of HPV-DNA ISH as an adjunct to p16 testing in oropharyngeal cancer. *Future Sci OA* 2020; 6: Fso606.
26. Zheng Z, Pan J, Chu B, *et al.* Downregulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. *Hum Pathol* 1999; 30: 458–66.
27. Tsai ST, Jin YT, Su JJ. Expression of EBER1 in primary and metastatic nasopharyngeal carcinoma tissues using in situ hybridization. A correlation with WHO histologic subtypes. *Cancer* 1996; 77: 231–6.
28. Liu H, Xia Y, Cui N. Impact of diabetes mellitus on treatment outcomes in patients with nasopharyngeal cancer. *Med Oncol* 2006; 23: 341–6.
29. Kanno M, Narita N, Fujimoto Y, *et al.* Third epidemiological analysis of nasopharyngeal carcinoma in the central region of Japan from 2006 to 2015. *Cancers (Basel)* 2019; 11: 1180.
30. Bertoni AG, Saydah S, Brancati FL. Diabetes and the risk of infection-related mortality in the U.S. *Diabetes Care* 2001; 24: 1044–9.
31. Doney R, Iqbal A, Heller SR, *et al.* A bittersweet response to infection in diabetes; targeting neutrophils to modify inflammation and improve host immunity. *Front Immunol* 2021; 12: 678771.
32. Tabuchi K, Nakayama M, Nishimura B, Hayashi K, Hara A. Early detection of nasopharyngeal carcinoma. *Int J Otolaryngol* 2011; 2011: 638058.
33. Yu MC, Nichols PW, Zou XN, Estes J, Henderson BE. Induction of malignant nasal cavity tumours in Wistar rats fed Chinese salted fish. *Br J Cancer* 1989; 60: 198–201.
34. Zheng X, Luo Y, Christensson B, Drettner B. Induction of nasal and nasopharyngeal tumours in Sprague-Dawley rats fed with Chinese salted fish. *Acta Otolaryngol* 1994; 114: 98–104.
35. Wang L, Bai YY, Yang Y, *et al.* Diabetes mellitus stimulates pancreatic cancer growth and epithelial-mesenchymal transition-mediated metastasis via a p38 MAPK pathway. *Oncotarget* 2016; 7: 38539–50.
36. Yen YC, Lin C, Lin SW, *et al.* Effect of metformin on the incidence of head and neck cancer in diabetics. *Head Neck* 2015; 37: 1268–73.
37. Sun XS, Xie SY, Luo DH, *et al.* Impact of smoking on survival in nasopharyngeal carcinoma: a cohort study with 23,325 patients diagnosed from 1990 to 2016. *Radiother Oncol* 2021; 162: 7–17.
38. OuYang PY, Su Z, Tang J, *et al.* Diabetes, prediabetes and the survival of nasopharyngeal carcinoma: a study of 5,860 patients. *PLoS One* 2014; 9: e111073.
39. Wu SS, Chen B, Fleming CW, *et al.* Nasopharyngeal cancer: incidence and prognosis of human papillomavirus and Epstein-Barr virus association at a single North American institution. *Head Neck* 2022; 44: 851–61.
40. Huang SH, Jacinto JCK, O'Sullivan B, *et al.* Clinical presentation and outcome of human papillomavirus-positive nasopharyngeal carcinoma in a North American cohort. *Cancer* 2022; 128: 2908–21.
41. Lee DY, Lee TS. Associations between metabolic syndrome and gynecologic cancer. *Obstet Gynecol Sci* 2020; 63: 215–24.
42. Maxwell JH, Kumar B, Feng FY, *et al.* HPV-positive/p16-positive/EBV-negative nasopharyngeal carcinoma in white North Americans. *Head Neck* 2010; 32: 562–7.
43. Lin Z, Khong B, Kwok S, *et al.* Human papillomavirus 16 detected in nasopharyngeal carcinomas in white Americans but not in endemic Southern Chinese patients. *Head Neck* 2014; 36: 709–14.
44. Stenmark MH, McHugh JB, Schipper M, *et al.* Nonendemic HPV-positive nasopharyngeal carcinoma: association with poor prognosis. *Int J Radiat Oncol Biol Phys* 2014; 88: 580–8.
45. Shimizu Y, Murakami N, Mori T, *et al.* Clinical impact of p16 positivity in nasopharyngeal carcinoma. *Laryngoscope Investig Otolaryngol* 2022; 7: 994–1001.