Prokineticins and Merkel cell polyomavirus infection in Merkel cell carcinoma

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Background: Prokineticin-1 (PROK1) and prokineticin-2 (PROK2) are chemokine-like proteins that may influence cancer growth by regulating host defence and angiogenesis. Their significance in viral infection-associated cancer is incompletely understood. We studied prokineticins in Merkel cell carcinoma (MCC), a skin cancer linked with Merkel cell polyomavirus (MCPyV) infection.

Methods: Carcinoma cell expression of PROK1 and PROK2 and their receptors (PROKR1 and PROKR2) was investigated with immunohistochemistry, and tumour PROK1 and PROK2 mRNA content with quantitative PCR from 98 MCCs. Subsets of tumour infiltrating leukocytes were identified using immunohistochemistry.

Results: Merkel cell polyomavirus-positive MCCs had higher than the median PROK2 mRNA content, whereas MCPyV-negative MCCs contained frequently PROK1 mRNA. Cancers with high tumour PROK2 mRNA content had high counts of tumour infiltrating macrophages (CD68 + and CD163 + cells). Patients with higher than the median PROK2 mRNA content had 44.9% 5-year survival compared with 23.5% among those with a smaller content (hazard ratio (HR): 0.53; 95% confidence interval (CI): 0.34–0.84; $P = 0.005$), whereas the presence of PROK1 mRNA in tumour was associated with unfavourable survival ($P = 0.052$).

Conclusions: The results suggest that prokineticins are associated with MCPyV infection and participate in regulation of the immune response in MCC, and may influence outcome of MCC patients.

Prokineticins comprise a family of chemokine-like proteins that are highly conserved across species. The family consists of only two ligands, prokineticin-1 (PROK1) and prokineticin-2 (PROK2), and of only two homologous G protein-coupled receptors, the prokineticin receptor-1 (PROKR1) and prokineticin receptor-2 (PROKR2) (Monnier and Samson, 2010). Prokineticin-1 and PROK2 were first identified as peptides that contract the gastrointestinal smooth muscle at a subnanomolar range (Li et al, 2001). The prokineticin family was later found to influence a great diversity of biological functions and to participate in coordination of complex physiological activities, such as feeding, drinking, regulation of the circadian rhythm, and hyperalgesia, and they have been suggested to be involved in angiogenesis, inflammation, and cancer (Monnier and Samson, 2010).

Prokineticin-1 and PROK2 mediate inflammatory reactions (Denison et al, 2008; Giannini et al, 2009, Watson et al, 2012). Prokineticin-2 is preferentially expressed by the innate immune cells, such as neutrophils, monocytes, and macrophages. Prokineticin-1 is less specifically expressed in immune cells, but it regulates monocyte differentiation and activation (Dorsch et al, 2005; Monnier and Samson, 2008). Inflammation-induced PROK2 expression stimulates haematopoiesis, haematopoietic cell mobilisation, chemotaxis, and activation and differentiation of myeloid cells (LeCouter et al, 2004; Martucci et al, 2006). Myeloid cell-derived PROK2 may stimulate also tumour angiogenesis (Shojaei et al, 2007). Like vascular endothelial growth factor (VEGF), PROK1 and PROK2 can sequester to the extracellular matrix, and the promoter regions of the genes contain hypoxia inducible

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factor-1 (HIF-1) binding sites that are activated at low oxygen levels (LeCouter *et al*, 2001, 2003). Prokineticins might thus influence tumour growth either by induction of angiogenesis or by influencing host defence.

Little is known about the role of prokineticins in human cancer, and to our knowledge their significance in viral infection-associated cancers has not been investigated. We chose to study prokineticins in Merkel cell carcinoma (MCC), a rare type of skin cancer, since this cancer is likely frequently caused by viral infection (Feng *et al*, 2008) and the host defence may influence its natural course (Sihto *et al*, 2012). Merkel cell carcinoma affects mainly elderly and immunocompromised individuals, such as patients infected with human immunodeficiency virus (HIV) or recipients of organ transplants (Engels *et al*, 2002; Lanoy *et al*, 2010; Tadmor *et al*, 2011). The majority of MCCs (70–80%) harbour Merkel cell polyomavirus (MCPyV) DNA that is clonally integrated into the MCC genome (Feng *et al*, 2008; Sihto *et al*, 2009a). Merkel cell polyomavirus-positive MCCs express a major viral oncoprotein, the large T antigen that has binding sites for key tumour suppressor proteins, the retinoblastoma protein and p53 (Shuda *et al*, 2008; Houben *et al*, 2009). Analogous to human papilloma virus (HPV)-associated oropharyngeal cancer, patients with MCPyV-positive MCC have been reported to be associated with more favourable survival than those with MCPyV-negative cancer (Sihto *et al*, 2009a; Ang *et al*, 2010; Bhatia *et al*, 2010). Merkel cell carcinoma patients with high intratumoural T lymphocyte counts appear to have better outcome than those with a low count (Paulson *et al*, 2011; Sihto *et al*, 2012). In one study, high tumour T-cell counts were associated with favourable survival regardless of the tumour MCPyV status (Sihto *et al*, 2012). A few case reports have described spontaneous regression of MCC, likely associated with a local inflammation reaction at the tumour site (Richetta *et al*, 2008; Ciudad *et al*, 2010; Wooff *et al*, 2010).

In this study, we investigate the expression of PROK1, PROK2, PROKR1, and PROKR2 in MCC, and their associations with cancer histopathological and clinical features, the presence of MCPyV infection in tumour, the types of tumour infiltrating leukocytes, intratumoural blood vessel counts, and patient survival. To the best of our knowledge, this is the first study that addresses the role of prokineticins in human cancer frequently associated with viral infection.

MATERIALS AND METHODS

Patients and tissue samples. Patients diagnosed with MCC in Finland in 1979–2004 were searched from the files of the Finnish Cancer Registry (Teppo *et al*, 1985) and from the patient registry of the Helsinki University Central Hospital (the largest hospital in Finland). The diagnoses were reviewed by a pathologist with a special interest in MCC (TB). The diagnosis was considered as confirmed when tumour morphology was compatible with MCC and tumour cells showed cytokeratin-20 expression, or when cytokeratin-20 was not expressed both synaptophysin and chromogranin A immunostainings were positive. Immunostaining for the thyroid transcription factor-1 was required to be negative to exclude metastatic small cell lung carcinoma. Of the 207 patients identified in this nation-wide search, 109 cases (52.7%) were not available for study for the following reasons: formalin-fixed paraffin-embedded (FFPE) tissue was not available ($n = 37$), cancer was not MCC when histology was reviewed ($n = 13$), the primary tumour site was unknown ($n = 8$), clinical information was not available ($n = 16$), an adequate amount of tumour tissue was unavailable for RNA extraction ($n = 30$), or the quality of the extracted RNA was unsatisfactory ($n = 5$). The remaining 98 patients were included in the study.

Tumour histology was classified according to the World Health Organization (WHO) Criteria (Kohler and Kerl, 2006; Sihto *et al*, 2009a). Clinical staging was done according to Lemos *et al* (2010). The longest tumour diameter was measured from the haematoxylin eosin-stained slides, and whenever this was not possible (large tumours), the diameter was extracted from the case records. The patients were treated with surgical excision of the primary tumour. In addition, 13 (13.2%) patients received postoperative external beam radiation therapy. None of the patients was treated with systemic adjuvant or neoadjuvant chemotherapy.

This study was performed according to the principles in the declaration of Helsinki. An institutional review board of the Helsinki University Central Hospital approved the study. A permission to collect cancer tissue and clinical data for the study purposes was provided by the Ministry of Health and Social Affairs, Finland.

Immunohistochemistry. Expression of PROK1, PROK2, PROKR1, PROKR2, Ki-67, retinoblastoma protein, large T antigen, p53, and CD31 was analysed using immunohistochemistry from a tissue microarray. The numbers of intratumoural CD3 + cells (T lymphocytes), CD4 + cells (helper T cells), CD8 + cells (cytotoxic T cells), FoxP3 + cells (regulatory T cells), CD16 + cells (small CD16 + cells representing mainly natural killer cells and granulocytes, large CD16 + cells macrophages), CD68 + cells (macrophages), and CD163 + cells (macrophages) were analysed using immunohistochemistry from whole tumour tissue sections. The immunostaining methods for Ki-67, retinoblastoma protein, p53, CD3, CD4, CD8, FoxP3, CD16, CD31, CD68, CD163, and the large T antigen have been described elsewhere (Sihto *et al*, 2009b, 2011, 2012; Waltari *et al*, 2011).

The mean number of tumour infiltrating immune cells was counted from three tumour regions with most abundant immune cell infiltration per one high-power field (HPF) using $\times 400$ magnification of the microscope (Olympus BX50, Center Valley, PA, USA). Only stained cells with visible nuclei and located in tumour were counted (Sihto *et al*, 2012).

To immunostain PROK1, PROK2, PROKR1, and PROKR2, $5 \mu m$ sections were cut from a tumour microarray, deparaffinised by two changes of xylene for 5 min, and then rehydrated through a decreasing ethanol gradient. Subsequently, antigen retrieval was done in 10 mM citrate buffer at pH 6.0, heated in a water bath at 98 °C for 40 min and then let to rest at room temperature for 20 min. Endogenous peroxidase was blocked by incubating in 1% hydrogen peroxide at room temperature for 30 min. The sections were blocked with a Powervision pre-antibody blocking solution for 10 min at room temperature before overnight incubation with the primary antibodies diluted in the same solution at 4 °C. The antibodies used in staining were a rabbit polyclonal anti-PROK1, a rabbit polyclonal anti-PROK2 (CVL-PAB0190-0 and CVL-PAB0409-0, respectively; both diluted 1 : 200; Covalab, Villeur-banne, France), a rabbit polyclonal anti-PROKR1 antibody, and a rabbit polyclonal anti-PROKR2 antibody (LS-C66877 and LS-C66878, respectively; Lifespan Biosciences Inc., Seattle, WA, USA, diluted 1 : 300). Binding of the primary antibodies was detected with a Histofine anti-rabbit simple stain MAX PO kit (Nichirei Biosciences Inc., Tokyo, Japan). Histologically normal human testis and wound tissue served as positive controls.

The immunohistochemical stains for PROK1, PROK2, PROKR1, and PROKR2 were assessed semiquantitatively using $\times 200$ magnification (Olympus BX50 microscope); photographs were taken with a Leica DM LB microscope (Leica Microsystems GmbH, Wetzlar, Germany) and with an Olympus DP50 colour camera. The stains were classified negative (0) when no staining was present on carcinoma cells, positive (+) when most carcinoma cells showed light to moderate staining intensity, and

strongly positive (+ +) when most carcinoma cells showed strong staining intensity.

Tumour blood vessels counts. Intratumoural microvascular density was evaluated with the criteria described earlier (Sihto *et al*, 2009b). CD31-positive vascular structures containing a clearly visible lumen were counted from three HPFs using × 400 magnification of the microscope (Olympus BX50). At least two representative tissue microarray spots were counted from each tumour. The vascular structure was counted as a single vessel whenever it was separated from an adjacent vessel by intervening tissue.

RNA extraction. Three 10-μm-thick tissue sections were cut from each FFPE tumour, and the slices were lysed overnight at 56 °C using a MoleStrips RNA Tissue kit (MGK30.101-101; Mole Genetics AS, Lysaker, Norway). The lysis buffer was supplemented with 10 μg μl^{-1} of proteinase K (Roche, Basel, Switzerland) before overnight incubation. After deparaffinisation, RNA was extracted from the lysate using MoleStrips kits and an automated nucleic acid extraction instrument (GeneMole; Mole Genetics AS). The yield varied between 4 and 68 ng μl^{-1} of pure RNA. Sample RNA concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time quantitative PCR analysis. Merkel cell polyomavirus DNA was detected by quantitative PCR (qPCR) as described earlier (Sihto *et al*, 2009a). In brief, MCPyV DNA was quantified using a primer pair (forward: 5′-GCATCTGCACCTTTTCTAGACTC-3′; reverse: 5′-TTTTGCCTTATAGACTTTTCCATATCT-3′) and a hydrolysis probe 6 (5′-TTCCTCTG-3′) with a Lightcycler 480 instrument (Roche Diagnostics, Mannheim, Germany) from extracted DNA. Each PCR product was sequenced using BigDye3 termination chemistry and an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

To measure tumour PROK1 and PROK2 content, ~60–200 ng of extracted RNA was first reverse transcribed into cDNA in a 20-μl reaction using a Superscript VILO cDNA synthesis kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Sample PROK1 mRNA expression was measured using a forward primer 5′-CCACGCGAGTCTCAATCA-3′ and a reverse primer 5′-ACTGGACATCCCGCTCAC-3′, and PROK2 (Bv8 homologue, variant 1) mRNA expression with a forward primer 5′-AAGCAAA AGGAAAAAGGAGGTT-3′ and a reverse primer 5′-CAGACATG GGCAAGTGTGA-3′. Locked nucleic acid hydrolysis probes 63 and 88 from the Universal ProbeLibrary (Roche Diagnostics) were used for PROK1 and PROK2 assays, respectively. Quantitative PCR measurements were done with a Lightcycler 480 instrument (Roche Diagnostics). The PCR mixture, made with the Lightcycler Probes Master reagents (Roche Diagnostics), contained 1 × ProbeMaster, 100 nM probe, 200 nM of each primer, and 2 μl of template cDNA. The primers and the probe were designed with the Probefinder software at the Universal ProbeLibrary Assay Design Center (Roche Diagnostics; http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_00000). The Lightcycler qPCR program consisted of initial denaturation for 10 min at 95 °C, followed by 45 cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 s.

The expression levels were quantified relative to a housekeeping gene encoding the TATA-box-binding protein (*TBP*, GenBank #M55654) in the same samples (Universal ProbeLibrary human TBP gene assay; Roche Diagnostics). *TBP* was chosen, because it is one of genes that have most constant expression across different human tissues (Radonic *et al*, 2004). RNA from histologically normal human testis and from human peripheral blood leukocytes was used as a positive control for tumour PROK1 and PROK2 content measurements, respectively. A standard curve was applied to determine the real amplification efficiency, and an inter-plate calibrator sample was used for normalisation between different measurements. All samples were run in triplicates. The relative quantity of the target was calculated with the E-method software (Roche Diagnostics). A qPCR mixture was run on a 2.5% agarose gel to verify that a single amplicon was produced in each analysis and that the amplicon had the correct size (84 nt for PROK1 and 64 nt for PROK2).

Statistical methods. Frequency tables were analysed with the χ^2 test or Fisher's exact test. Variable distributions between groups were compared with the Mann–Whitney test, and correlation between continuous variables was analysed by computing the Spearman rank correlation. Survival was analysed using the Kaplan–Meier method, and survival between groups was compared using the log-rank test. Overall survival was calculated from the date of the diagnosis to the date of death censoring patients alive on the last day of follow-up (1 August 2008). Merkel cell carcinoma-specific survival was calculated from the date of the diagnosis to the date of death, considered to be caused by MCC based on clinical judgment or autopsy evidence censoring individuals alive and those who died from an intercurrent cause. The hazard ratios (HRs) and their confidence intervals (CIs) were calculated with a univariable Cox's proportional hazard model. Multivariable survival analysis was carried out with a Cox's proportional hazard model. The statistical analyses were done using the SPSS Statistics 20 software (IBM, Armonk, NY, USA).

RESULTS

MCCs express frequently PROK2 and PROKR2. In immuno-stainings, PROK2 and PROKR2 expression was more frequent in carcinoma cells than PROK1 or PROKR1 expression. Because strong (+ +) expression was uncommon (2% of the tumours), PROK1, PROKR1, PROK2, and PROKR2 expression were considered as either negative (−) or positive (+) in further analyses (Table 1; Figure 1).

Carcinoma cell PROK1 and PROK2 expression correlated strongly, and their expression correlated also with expression of their receptors. In the subset of tumours where both PROK1 and PROK2 expression could be assessed (90 out of 98 tumours), 19 (90.5%) out of the 21 PROK1-positive MCCs were also PROK2 positive as compared with 22 (31.9%) of the 69 PROK1-negative tumours ($P < 0.001$). Expression of PROK1 was strongly associated also with PROKR1 expression (7 (87.5%) out of the 8 tumours that expressed PROKR1 expressed also PROK1 compared with 13 (16.5%) of the 79 tumours that were PROKR1 negative, $P < 0.001$), and less strongly with PROKR2 expression (14 (31.1%) out of the

Table 1. Expression of prokineticin-1, prokineticin-2, and their receptors in Merkel cell carcinoma

Prokineticin ligand or receptor[a]	Expression in immunohistochemistry	
	Negative (−), N (%)	Positive (+), N (%)
Prokineticin-1	71 (77.2)	21 (22.8)
Prokineticin-2	49 (54.4)	41 (45.6)
Prokineticin receptor-1	80 (90.1)	8 (9.9)
Prokineticin receptor-2	45 (48.9)	47 (51.1)

[a]Tissue sample was not available in six, eight, ten, and six cases for immunostaining of prokineticin-1, prokineticin-2, prokineticin receptor-1, and prokineticin receptor-2, respectively.
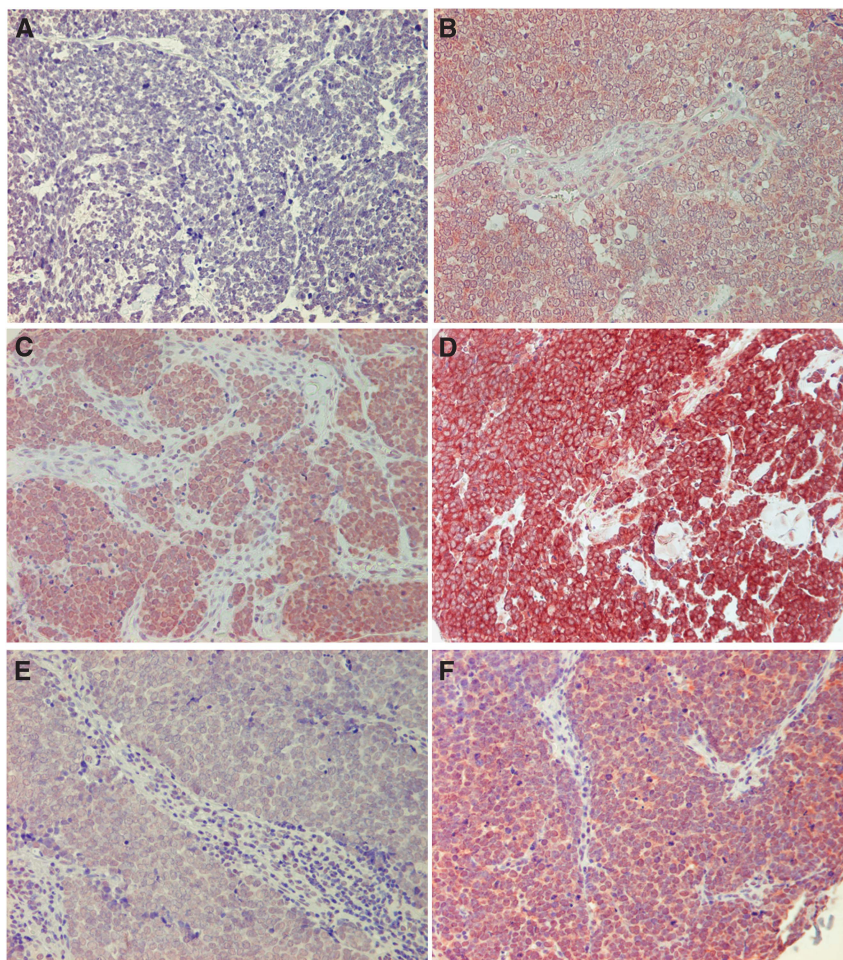
Figure 1. Examples of PROK1, PROK2, PROKR1, and PROKR2 immunostainings on tumour tissue microarrays. (A) MCC with no PROK1 expression ($-$), (B) moderate tumour PROK1 expression ($+$), (C) moderate PROK2 expression ($+$), (D) strong PROK2 expression ($++$), (E) weak PROKR1 expression ($+$), and (F) moderate PROKR2 expression ($+$). In (B–F) carcinoma cells stain positively, whereas the stromal tissue including tumour infiltrating leukocytes are negative. Most carcinoma cell immunostaining is cytoplasmic, but occasionally also membranous staining is present (B, D). Magnification $\times 200$.

45 tumours that expressed PROKR2 expressed PROK1 compared with 6 (13.6%) out of the 44 tumours that were PROKR2 negative, $P = 0.048$). Similarly, PROK2 expression was associated with PROKR1 expression (all eight tumours that expressed PROKR1 expressed also PROK2, whereas 33 (41.8%) of the 79 tumours that did not express PROKR1 expressed PROK2, $P = 0.002$) and with PROKR2 expression (29 (64.4%) of the 45 tumours that expressed PROKR2 expressed also PROK2 as compared with 12 (27.9%) of the 43 tumours that were PROKR2 negative, $P < 0.001$). Carcinoma cell PROKR2 immunoexpression was significantly associated with the presence of MCPyV DNA in MCCs ($P = 0.007$), expression of MCPyV large T antigen ($P = 0.005$, Figure 2), and expression of the retinoblastoma protein in tumour ($P = 0.030$).

High tumour PROK2 mRNA content is associated with the presence of MCPyV DNA and viral large T antigen. We next investigated whether tumour PROK1 or PROK2 ligand mRNA content measured with qPCR is associated with the clinical and molecular features of MCC. Since most MCCs (76 (77.6%) out of the 98 MCCs studied) did not contain detectable amounts of PROK1 mRNA, we classified tumour PROK1 mRNA content as either absent or present (the target gene to the reference gene ratio ranged from 0 to 1.55; median, 0). In contrast, all MCCs had detectable PROK2 mRNA (the target gene to the reference gene ratio ranged from 0.02 to 4.02; median, 0.571). Higher than the
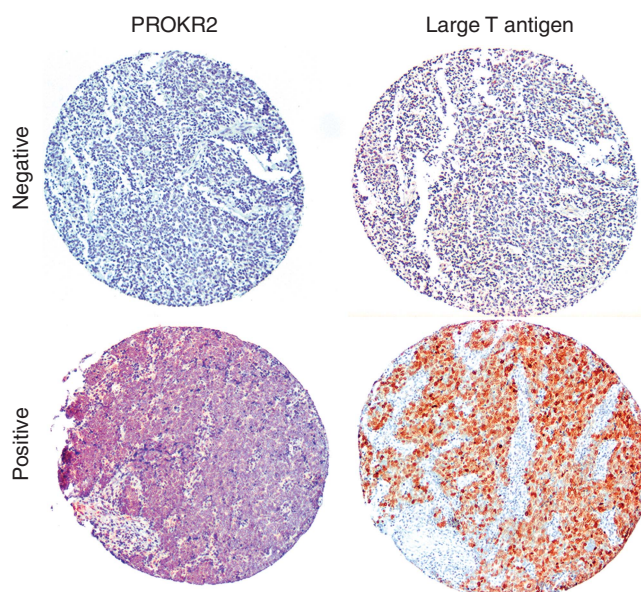

Figure 2. Adjacent tissue sections of Merkel cell carcinoma stained for PROKR2 and Merkel cell polyomavirus large T antigen by immunohistochemistry. Magnification $\times 100$.

median tumour PROK2 mRNA content was significantly ($P < 0.01$) associated with a low cell proliferation rate, the presence of MCPyV DNA, and expression of the viral large T antigen and the retinoblastoma protein, whereas the presence of PROK1 mRNA in tumour was significantly associated with the absence of MCPyV DNA, and the absence of MCPyV large T antigen and retinoblastoma protein expression (Table 2). Higher than the median tumour PROK2 content tended to associate with the absence of tumour p53 expression ($P = 0.087$), and was strongly associated with MCC localisation in a limb as compared with the trunk or the head and neck region ($P < 0.001$). Merkel cell carcinomas arising in a limb have been reported to be frequently MCPyV associated (Sihto et al, 2009a). Patients older than median (79 years) had frequently detectable PROK1 mRNA in tumour ($P = 0.041$), and the presence of PROK1 mRNA tended to associate with tumour p53 expression ($P = 0.056$).

Tumour PROK2 and PROKR2 expression is associated with several subsets of tumour infiltrating leukocytes. High viral large T antigen expression in MCC was associated with higher than median number of CD3 + cells ($P = 0.034$), CD4 + cells ($P = 0.043$), and CD68 + cells ($P = 0.027$), and also tended to be associated with higher than median number of small CD16 + cells ($P = 0.083$).

Tumour cell PROK2 and PROKR2 expression detected by immunohistochemistry was associated with the presence of some tumour infiltrating leukocyte subtypes (Table 3). Expression of PROK2 was associated with higher than the median (3.3/HPF) number of tumour infiltrating CD8 + cells (cytotoxic T cells, $P = 0.030$, Figure 3), and tended to be associated with higher than the median (3.7/HPF) number of CD163 + cells (macrophages, $P = 0.062$), and PROKR2 expression with higher than the median (4.7/HPF) number of tumour CD3 + cells (T lymphocytes, $P = 0.055$). The strongest association was, however, found between PROKR2 expression and the number of tumour infiltrating small CD16 + cells (natural killer cells). In all, 25 (71.4%) of the 35 MCCs that had higher than the median number of small CD16 + cells expressed PROKR2 as compared with 14 (33.3%) of the 42 tumours that contained the median number or fewer small CD16 + cells ($P = 0.001$, Figure 3). Cancer PROK2 or PROKR2 immunoexpression was not significantly associated with tumour infiltrating helper T cell (CD4 +) or regulatory T-cell (FoxP3 +) counts ($P > 0.10$ for each comparison). No significant associations were found between carcinoma cell PROK1 or PROKR1 expression and any of the tumour infiltrating immune cell subsets, although tumour PROK1 expression tended to be associated with higher than the median (5.3 cells/HPF) number of tumour infiltrating CD68 + cells (macrophages, $P = 0.054$).

Merkel cell carcinomas with higher than the median PROK2 mRNA content had higher than the median numbers of tumour infiltrating macrophages (CD68 + and CD163 + cells). The strongest association was found with the CD163 + cells; 29 (59.2%) of the 49 MCCs that had higher than the median PROK2 mRNA content had higher than the median count of CD163 + cells as compared with only 17 (34.7%) of the 49 tumours that contained lower than the median PROK2 mRNA content ($P = 0.015$). Similar associations were not found between tumour PROK1 mRNA content and the subsets of tumour infiltrating leukocytes investigated.

Tumour PROK1 and PROK2 mRNA content does not correlate with microvascular density. Since PROK1 and PROK2 are considered to promote angiogenesis, we counted the microvascular density of the tumour samples and correlated the count with tumour PROK1 and PROK2 mRNA contents. Intratumoural CD31-positive blood vessel counts ranged from 0 to 27.5 vessels/HPF in the 62 MCCs with tissue available for evaluation (median, 6.5; Supplementary Figure 1). Neither tumour PROK1 nor PROK2

mRNA content was significantly associated with tumour microvascular density counts regardless of whether the vessel counts were treated as continuous variables or variables categorised with the medians (data not shown; $P > 0.10$ for each comparison). The microvessel counts tended to be higher in MCPyV DNA-negative MCCs as compared with MCPyV DNA-positive cancers (median, 8.3/HPF vs 6.5/HPF, $P = 0.086$), but no difference was found in the microvessel counts between large T antigen-positive and -negative MCCs ($P = 0.846$).

High tumour PROK2 mRNA content is associated with favourable survival. Patients whose tumour contained higher than the median amount of PROK2 mRNA had 44.9% 5-year overall survival as compared with 23.5% 5-year survival among those with ⩽ median tumour PROK2 mRNA content (HR 0.53, 95% CI 0.34–0.84; $P = 0.005$), whereas the presence of PROK1 mRNA in tumour tended to be associated with unfavourable survival (5-year survival 20.0% vs 38.2% when PROK1 mRNA was absent; HR 1.61, 95% CI 0.99–2.79; $P = 0.052$). Higher than the median tumour PROK2 mRNA content was significantly associated also with MCC-specific survival (Figure 4). Expression of PROK1, PROK2, or their receptors in tumour cells in immunohistochemistry was not significantly associated with survival in univariable survival analyses ($P > 0.10$ for each analysis).

The independence of PROK1 and PROK2 mRNA content as prognostic factors was studied in a Cox multivariable model together with factors that were significantly associated with survival in a univariable survival analysis. The covariates entered to the model with PROK1 (tested present vs absent) and PROK2 ($<$ median vs $>$ median) mRNA content were age at diagnosis (as a continuous variable), tumour stage (1, 2 vs 3, four patients with distant metastases were excluded), the presence of MCPyV DNA in tumour (present vs absent), and tumour CD3 + cell count ($<$ median vs $>$ median). Neither PROK1 mRNA content (HR 0.77, 95% CI 0.38–1.59, $P = 0.48$) nor PROK2 content (HR 0.59, 95% CI 0.31–1.11, $P = 0.104$) influenced overall survival, whereas tumour stage (HR 3.46, 95% CI 1.58–7.58, $P = 0.002$), age (HR 1.05, 95% CI 1.02–1.09, $P < 0.001$), the presence of MCPyV DNA (HR 0.54, 95% CI 0.30–0.95, $P = 0.033$), and CD3 + cell count (HR 0.53, 95% CI 0.30–0.92, $P = 0.012$) turned out to be independent prognostic factors. Yet, PROK2 mRNA content was an independent factor (HR 0.53, 95% CI 0.29–0.99, $P = 0.047$) when the MCPyV DNA status was excluded from the multivariate analysis, likely reflecting the strong association between tumour PROK2 content and MCPyV positivity.

DISCUSSION

Immune microenvironment may influence MCC progression (Paulson et al, 2011; Sihto et al, 2012). In the present study, higher than the median MCC mRNA content of PROK2 was associated with lower than the median rate of tumour cell proliferation, the presence of MCPyV DNA and viral large T antigen in tumour, high tumour infiltrating macrophage counts (CD68 + and CD163 + cells), and favourable survival. In contrast, the presence of PROK1 mRNA in MCC was associated with the absence of MCPyV DNA and viral large T antigen, and with poor survival. Prokineticin-1 and PROK2 were not independent prognostic factors in a multivariate analysis suggesting that other immunological factors, such as T lymphocyte counts, may influence survival more strongly. When carcinoma cell ligand expression was studied with immunohistochemistry at the protein level, PROK1 and PROK2 expression was strongly associated with expression of their receptors (PROKR1 and PROKR2). Carcinoma cell PROK2 protein expression, in turn, was associated with high tumour CD8 + (cytotoxic T cells), and PROKR2 expression with

Table 2. Associations between tumour prokineticin-1 and prokineticin-2 mRNA contents and clinical and molecular factors in Merkel cell carcinoma

Factor	Tumour prokineticin-1 mRNA content			Tumour prokineticin-2 mRNA content		
	Absent	Present	P	≤Median	>Median	P
	N (%)	N (%)		N (%)	N (%)	
Age at diagnosis—years						
≤79 (median)	43 (86.0)	7 (14.0)		23 (46.0)	27 (54.0)	
>79	33 (68.7)	15 (31.3)	0.041	26 (54.2)	22 (45.8)	0.419
Gender						
Female	50 (74.6)	17 (25.4)		34 (50.7)	33 (49.3)	
Male	26 (83.9)	5 (16.1)	0.308	15 (48.4)	16 (51.6)	0.828
Tumour size—cm						
≤16.5 (median)	42 (85.7)	7 (14.3)		24 (49.0)	25 (51.0)	
>16.5	34 (69.4)	15 (30.6)	0.053	25 (51.0)	24 (49.0)	0.840
Stage						
I	30 (75.0)	10 (25.0)		19 (47.5)	21 (52.5)	
II	26 (76.5)	8 (23.5)		16 (47.1)	18 (52.9)	
III or IV	9 (75.0)	3 (25.0)	0.988	8 (66.7)	4 (33.3)	0.461
NA	12			12		
Tumour site						
Head or neck	39 (76.5)	12 (23.5)		33 (64.7)	18 (35.3)	
Limb	27 (79.4)	7 (20.6)		6 (23.5)	26 (76.5)	
Trunk	10 (76.9)	3 (23.1)	0.949	8 (61.5)	5 (38.5)	<0.001
Ki-67 expression—%						
≤56.4 (median)	38 (79.2)	10 (20.8)		16 (33.3)	32 (66.7)	
>56.4	36 (75.0)	12 (25.0)	0.627	32 (68.1)	15 (31.9)	0.001
NA	2			2		
p53 expression						
Not present	46 (83.6)	9 (16.4)		24 (43.4)	31 (56.6)	
Present	26 (66.7)	13 (33.3)	0.056	24 (61.5)	15 (38.5)	0.087
NA	4			4		
Tumour MCPyV DNA						
Not present	12 (57.1)	9 (42.9)		16 (76.2)	5 (23.8)	
Present	53 (81.5)	12 (18.5)	0.024	27 (41.5)	38 (58.5)	0.006
NA	12			12		
MCPyV large T antigen						
Not present	21 (63.6)	12 (36.4)		26 (78.8)	7 (21.2)	
Present	53 (85.5)	9 (14.5)	0.015	21 (33.9)	41 (66.1)	<0.001
NA	3			3		
Tumour Rb expression						
Not present	20 (64.5)	11 (35.5)		24 (77.4)	7 (22.6)	
Present	54 (83.1)	11 (16.9)	0.043	24 (36.9)	41 (63.1)	<0.001
NA	2			2		

Abbreviations: NA = not available; MCPyV = Merkel cell polyomavirus; Rb = retinoblastoma protein.

high small CD16+ (natural killer cell) counts and CD3+ (T lymphocyte) counts. Carcinoma cell PROKR2 expression was associated also with the presence of MCPyV DNA and large T antigen in tumour. Taken together, these results suggest that prokineticins are involved in the regulation of host immune response to MCC, are linked with MCPyV infection, and may influence survival of MCC patients.

We are unaware of any prior data on the prognostic role of PROK2 in human cancer, and at present relatively little is known about the clinical significance of the prokineticins in cancer. The information available suggests that their role may vary with the site and the type of cancer (Monnier and Samson, 2010). In line with the present results, PROK1 transcripts were detected in 31 (27%) out of 113 colorectal cancers investigated with qPCR, and patients with

Table 3. Associations between carcinoma cell prokineticin-2 and prokineticin receptor-2 protein expression and tumour infiltrating leukocyte subsets in Merkel cell carcinoma

	Prokineticin-2 expression			Prokineticin receptor-2 expression		
	Absent	Present	P	Absent	Present	P
Tumour infiltrating leukocyte subset	N (%)	N (%)		N (%)	N (%)	
CD3 +						
≤ Median (4.7/HPF)	27 (62.8)	16 (37.2)	0.129	26 (59.1)	18 (40.9)	0.055
> Median	20 (46.5)	23 (53.5)		17 (38.6)	27 (61.4)	
NA	12			10		
CD68 +						
≤ Median (5.3/HPF)	25 (55.6)	20 (44.4)	0.787	23 (48.9)	24 (51.1)	0.920
> Median	22 (52.4)	20 (47.6)		21 (50.0)	21 (50.0)	
NA	11			9		
CD4 +						
≤ Median (3.7/HPF)	24 (63.2)	14 (36.8)	0.311	22 (55.5)	18 (44.5)	0.285
> Median	18 (51.4)	17 (48.6)		14 (47.2)	19 (52.8)	
NA	25			25		
CD8 +						
≤ Median (3.3/HPF)	26 (66.7)	13 (33.3)	0.030	23 (57.5)	17 (42.5)	0.105
> Median	15 (41.7)	21 (58.3)		14 (38.9)	22 (61.1)	
NA	23			22		
FoxP3 +						
≤ Median (1.5/HPF)	23 (60.5)	15 (39.5)	0.356	22 (55.0)	18 (45.0)	0.303
> Median	19 (50.0)	19 (50.0)		16 (43.2)	21 (56.8)	
NA	22			21		
CD16 + (small cells)						
≤ Median (1.7/HPF)	25 (61.0)	16 (39.0)	0.278	28 (66.7)	14 (33.3)	0.001
> Median	17 (48.6)	18 (51.4)		10 (28.6)	25 (71.4)	
NA	22			21		
CD163 +						
≤ Median (3.7/HPF)	30 (63.8)	17 (36.2)	0.062	27 (55.1)	22 (44.9)	0.205
> Median	19 (44.2)	24 (55.8)		18 (41.9)	25 (58.1)	
NA	8			6		

Abbreviations: HPF = high-power field of the microscope; NA = not available.

PROK1 mRNA-positive cancer had poor survival as compared with those with PROK1 mRNA-negative colorectal cancer (Nagano *et al*, 2007). In neuroblastoma, PROKR1 transcript levels increased with advancing stage and were associated with metastasis, and functional studies showed that PROK1 activates the AKT pathway and induces neuroblastoma cell proliferation (Ngan *et al*, 2007). Prokineticin-1 mRNA levels are higher in prostate cancer (Pasquali *et al*, 2006), pancreatic cancer (Morales *et al*, 2007), and papillary thyroid cancer (Pasquali *et al*, 2011) tissue compared with the corresponding normal tissue, and PROK1 is a survival factor for human multiple myeloma cells (Li *et al*, 2010). On the other hand, PROK1 mRNA levels were higher in early-stage ovarian carcinomas than in late-stage cancers, and their levels did not correlate with the clinical outcome (Zhang *et al*, 2003), and in another study PROK1 transcripts were rarely detected in endometrial carcinoma (Ngan *et al*, 2006). In hepatocellular carcinoma, PROK2 expression decreased with increasing grade of malignancy and was found in Kupffer cells that are CD68-positive resident liver macrophages (Monnier *et al*, 2008). Regulation of prokineticins and their cellular distribution varies greatly from tissue to tissue and prokineticins have a wide range of tissue-specific biological activities (Monnier and Samson, 2010), which may be reflected in their variable roles in different types of human cancer.

Prokineticin-2 is the preferential prokineticin expressed by immune cells (Monnier and Samson, 2008), and only PROKR2 is detected in human neutrophils (Zhong *et al*, 2009). Prokineticin-2 promotes mobilisation of haematopoietic cells (Shojaei *et al*, 2007), neutrophil chemotaxis (Zhong *et al*, 2009), and triggers the release of pro-inflammatory cytokines from myeloid cells (Dorsch *et al*, 2005; Martucci *et al*, 2006; Monnier and Samson, 2008). Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) upregulate PROK2 in peripheral neutrophils and bone marrow cells, and interleukin 10 in monocytes and lymphocytes (Zhong *et al*, 2009). These findings are generally in agreement with the present results, since MCPyV-positive MCCs contained higher numbers of several types of immune cells compared with MCPyV-negative MCCs, such as CD3 + cells, CD8 + cells,
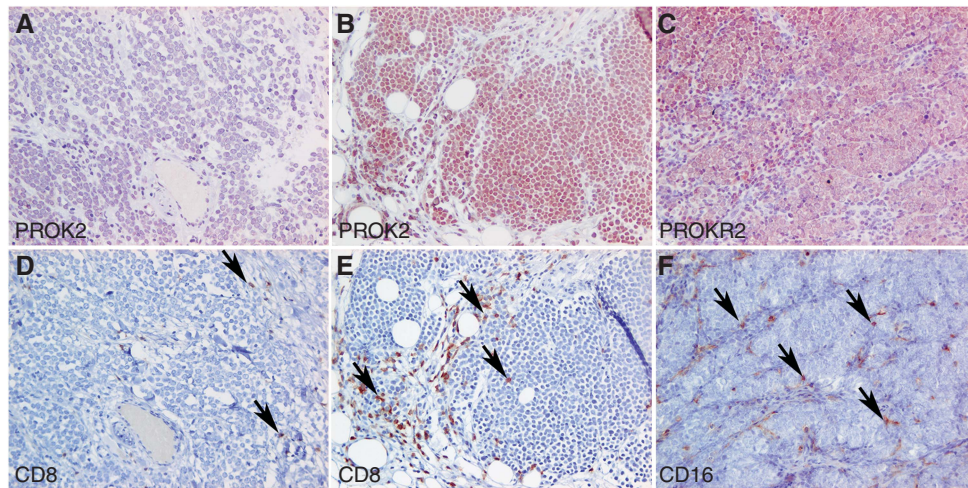
Figure 3. Examples of immunostainings for PROK2, PROKR2, CD8 and CD16 in pairs of closely spaced tissue sections (each of the panel pairs A and D, B and E, and C and F originate from one Merkel cell carcinoma). (**A**) Negative immunostaining for PROK2; (**B**) positive PROK2 staining in carcinoma cells; (**D**) a few positively staining CD8 + lymphocytes are present (arrows); (**E**) many CD8 + lymphocytes can be seen (arrows); (**C**) positive immunostaining for PROKR2 in carcinoma cells; (**F**) several CD16 + cells reside within the tumour stroma. Magnification × 200.
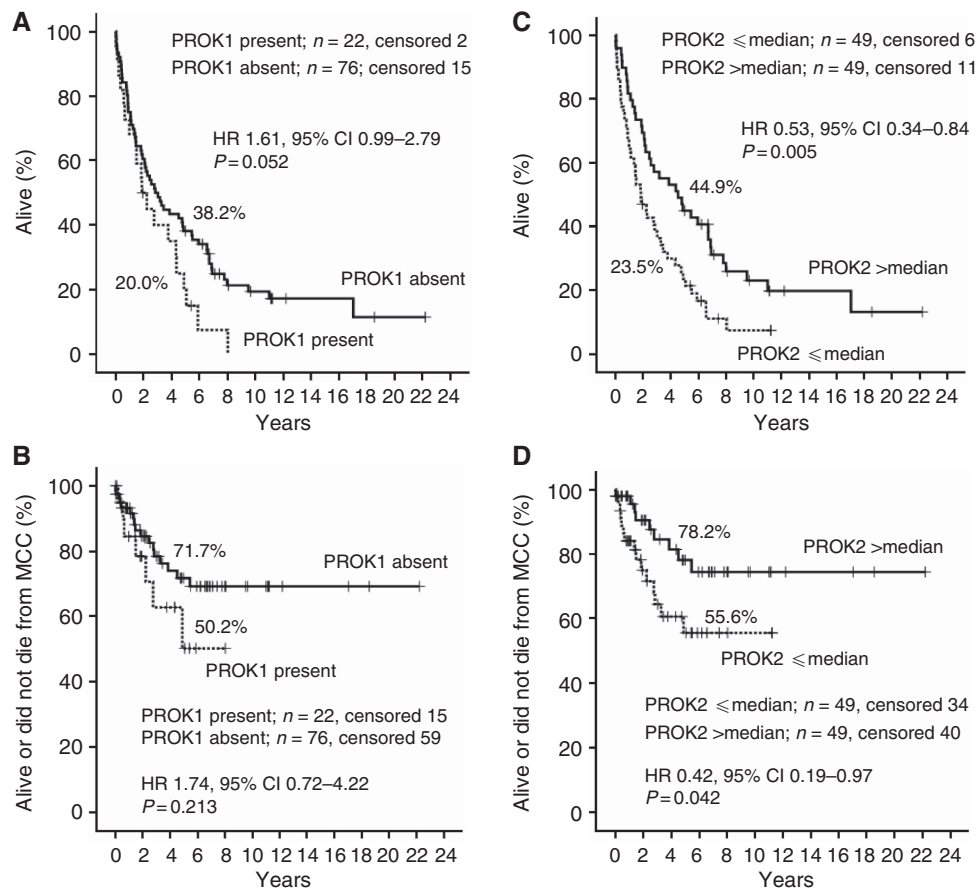
Figure 4. Associations between the primary tumour PROK1 and PROK2 mRNA contents and MCC-specific and overall survival in univariable survival analyses. (**A**) Influence of tumour PROK1 mRNA content on overall survival; (**B**) tumour PROK1 mRNA content on MCC-specific survival; (**C**) tumour PROK2 mRNA content on overall survival; (**D**) tumour PROK2 mRNA content on MCC-specific survival. Survival between groups was compared with the log-rank test, and the hazard ratios with a univariable Cox model. The patients censored are indicated with a bar. Five-year survival figures are provided.

CD16 + cells, FoxP3 + cells, and CD68 + cells, and MCC patients with high tumour infiltrating T-cell counts have more favourable survival than those with small counts (Sihto *et al*, 2012). Prokineticin-2 is thus likely involved in orchestrating the immune defence against the MCPyV infection in patients with MCPyV-related MCC, and the favourable prognosis associated with high tumour PROK2 content may be related to high numbers of tumour infiltrating immune cells. Tumour PROK1

content was associated with the absence of MCPyV infection and frequent p53 expression, which may in part account for the poor survival outcomes of the patients with detectable PROK1 in tumour.

Ample evidence indicates that prokineticins are involved in physiological angiogenesis (Monnier and Samson, 2010). They may participate also in neoplastic angiogenesis, possibly due to their proinflammatory role. For example, in mouse models PROK2 mediates myeloid cell-dependent angiogenesis via regulating mobilisation of CD11b + Gr-1 + myeloid cells that further secrete PROK2 (Shojaei et al, 2007, 2008). The effect of an anti-PROK2 antibody on tumour vasculature was additive to that of an anti-VEGF antibody suggesting concerted actions for PROK2 with VEGF (Shojaei et al, 2007). Yet, the proangiogenic properties of PROK2 may depend on the tissue type and conditions. In highly vascularised glioblastoma xenograft a PROK2 antagonist PKRA7 reduced endothelial cell branching, whereas in poorly vascularised pancreatic cancer tissue the anti-tumour effect of PKRA7 was mediated by blocking of myeloid cell infiltration and migration (Curtis et al, 2013). In the present series, neither tumour PROK1 nor PROK2 mRNA content was associated with tumour microvascular density when assessed from tissue microarray samples that may not be ideal for this purpose. Tumour microvascular density is likely regulated by many factors, and the role of PROK1 and PROK2 in angiogenesis of MCC requires further study.

In conclusion, we found that tumour PROK2 mRNA content is associated with the presence of MCPyV DNA in MCC, high numbers of tumour infiltrating macrophages and favourable survival, whereas PROK1 is associated with the absence of MCPyV, and tends to be associated with p53 expression and poor survival. These results suggest that prokineticins are involved in the host immune response to MCPyV. Further studies should be conducted to find out the role of PROK1 and PROK2 in other viral infection-associated cancers, such as cervical cancer or head and neck cancer related to HPV infection. The prognostic role of PROK2 remains unknown in cancer types that have not been linked with tumour viruses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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