Abstract

Purpose: Heterogeneous ribonucleoprotein K (hnRNP K) regulates thymidine phosphorylase (TP) mRNA stability. The aim of the present study was to analyze hnRNP K and TP expression in nasopharyngeal carcinoma (NPC) and to evaluate the prognostic and therapeutic potential of these two markers.

Experimental Design: We analyzed hnRNP K and TP expression immunohistochemically in 121 clinically proven NPC cases. Statistical analyses were applied to correlate cytoplasmic hnRNP K with elevated TP expression and determine the prognostic significance of these parameters. The therapeutic implication of elevated TP expression was determined by measuring sensitivity of NPC cells to the TP-targeting drug, 5-fluoro-5'-deoxyuridine (5'-DFUR).

Results: There was a high correlation between cytoplasmic hnRNP K and high TP ($P < 0.001$, respectively). Both cytoplasmic hnRNP K and high TP were associated with poor overall survival ($P = 0.007$ and $P < 0.001$, respectively) and distant metastasis-free survival ($P = 0.003$ and 0.001, respectively) of NPC patients. A multivariate analysis confirmed that both cytoplasmic hnRNP K and high TP are independent prognostic predictors for OS ($P = 0.020$ and 0.010, respectively). NPC cells expressing high TP were more sensitive to treatment with the TP-targeting drug, 5'-DFUR.

Conclusions: Cytoplasmic hnRNP K and high TP are associated with shorter OS and distant metastasis-free survival in NPC patients. In vitro experiments suggest that NPC tumors with high TP expression may be sensitive to 5'-DFUR treatment. Cytoplasmic hnRNP K and high TP may be potential prognostic and therapeutic markers for NPC, but additional validation studies are warranted.

Heterogeneous ribonucleoprotein K (hnRNP K) is a member of the hnRNP family of proteins, which directly interact with DNA and RNA through their K homology domains and are involved in regulating gene expression at multiple levels, including transcription, RNA splicing, and translation (1). hnRNP K may contribute to tumorigenesis by regulating the expression of the oncogenes c-myc and eukaryotic translation initiation factor 4E. hnRNP induces c-myc expression through the c-myc internal ribosome entry segment (2, 3) and activates eukaryotic translation initiation factor 4E by binding to a core polypyrimidine element in the eukaryotic translation initiation factor 4E promoter (4). hnRNP K is located primarily in the nucleus and must be translocated to cytoplasm to exert its translation-regulatory function (3). A recent report suggests that hnRNP K promotes migration of human fibrosarcoma cells, making it a potential target for metastasis therapy (5). To date, a limited number of clinical studies have shown that the expression of hnRNP K is aberrantly increased in certain cancer cells, including colorectal cancer (6), esophageal cancer (7), lung cancer (8), and oral squamous cell carcinoma (9) cells. Decreased expression of nuclear hnRNP K is associated with shorter survival in patients with Dukes C colorectal cancer (6), but its relationship to other prognostic parameters, including recurrence and metastasis, is not known.

A novel role for hnRNP K in the stabilization of gastrin mRNA has been described recently (10). This report is consistent with our unpublished observation that cytoplasmic hnRNP K increased the half-life of thymidine phosphorylase (TP) mRNA. TP, also designated platelet-derived endothelial cell growth factor or Gliostatin, plays an important role in...
nucleoside metabolism. Additionally, both angiogenic and chemotactic properties have been attributed to TP, which has also been shown to inhibit tumor cell apoptosis (reviewed in ref. 11). Importantly, the enzymatic activity of TP effectively metabolizes several clinically approved antitumor fluoropyrimidine prodrugs, such as doxifluridine and capecitabine, into their active forms. Thus, its increased activity in tumors may be exploited for cancer treatment (11). In various cancers, TP is overexpressed and is thus of prognostic value (12–21). The expression of TP in nasopharyngeal carcinoma (NPC), however, has not been studied, nor has that of its regulator, hnRNP K.

NPC is a rare malignancy in Caucasians but is relatively common in the southeastern region of China. According to 2002 data from the Cancer Registry of Taiwan, NPC is the ninth most common cancer with an estimated incidence of ~6 per 100,000. In specific provinces of southern China and Hong Kong, the incidence is as high as 13 to 30 per 100,000 in males and ~11 per 100,000 in females. NPC is generally sensitive to radiation therapy, but more advanced cases of the disease may require a combination of radiotherapy and chemotherapy. The survival rate among NPC patients treated according to current treatment regimes is ~92% at 1 year and ~50% at 5 years, with 20% to 25% of patients eventually developing distant metastases (22, 23). A need for improved or supplemental therapeutic strategies and better prognostic indicators clearly exists.

In the present study, we show that hnRNP K and its target, TP, are overexpressed in NPC tumor cells. Aberrant cytoplasmic localization of hnRNP K and overexpression of TP are associated with shorter overall survival (OS) and distant metastasis-free survival (DMFS). Multivariate analysis shows that cytoplasmic hnRNP K and high TP are independent prognostic indicators for patient survival. In addition, the sensitivity of NPC cells to the capecitabine intermediate prodruk, 5-fluoro-5-deoxyuridine (5'-DFUR), was enhanced in cells expressing elevated TP. Our findings strongly suggest that hnRNP K and its target, TP, are useful prognostic markers for NPC and may prove valuable in the design of effective therapeutic strategies.

Materials and Methods

Patients, clinical staging, treatment, and assessment of clinical outcome. This retrospective cohort comprises 121 NPC patients who had been admitted to Chang Gung Memorial Hospital at Lin-Kou from 1990 to 1999. Clinical stage was defined according to the 2002 cancer staging system revised by the American Joint Committee on Cancer. Histologic typing was done according to the WHO classification criteria as described previously (23). This study was reviewed and approved by the institutional review board and ethics committee of Chang Gung Memorial Hospital. Informed consent was obtained from all patients. All enrolled patients had been treated with definitive radiotherapy (cumulative dose of external beam radiotherapy > 60 Gy). Among them, 33 patients received additional chemotherapy in the Department of Medical Oncology at Chang Gung Memorial Hospital. Patients diagnosed with distant metastatic disease at presentation (M1 stage) and/or those who had undergone previous treatment at another institute were excluded from the present study. For all enrolled patients, pathology reports were obtained from pathologic databases and medical records and reviewed for confirmation of the NPC diagnosis (reviewed by C. Hsieh). Information on stage, treatment, and follow-up were collected from hospital tumor registries and medical files as was limited information on family history. The primary endpoint was OS, which was calculated from the date of diagnosis to the date of death or the last follow-up. Disease-free survival (DFS), DMFS, and local recurrence-free survival (LRFS) were also assessed. The time to local recurrence or distant metastasis was calculated using the date on which local recurrence or distant metastasis status was detected as the endpoint. Patients who died without occurrence of local recurrence or distant metastasis were censored in the analyses of DFS, LRFS, and DMFS.

Immunohistochemical staining analysis. Immunohistochemical analyses were done using an automatic immunohistochemistry staining device according to the manufacturer's instructions (Bond Vision BioSystems). Tissue sections were retrieved using Bond Epitope Retrieval Solution 1 on the Bond-max automated immunostainer (Vision BioSystems) and stained with antibodies to hnRNP K (mouse monoclonal antibody, 1:300 dilution; Santa Cruz Biotechnology) and TP (mouse monoclonal antibody, 1:500 dilution; Santa Cruz Biotechnology). A polymer detection system (Bond polymer refine, Vision BioSystems) was used to reduce nonspecific staining. Tissue sections were treated with liquid 3,3'-diaminobenzidine reagent using 3'-diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin as the counterstaining reagent. For analysis of cytoplasmic hnRNP K, a sample was defined as "cytoplasmic positive" in cases where >10% of the tumor cells exhibited cytoplasmic staining and as "cytoplasmic negative" where ≤10% of cells were stained. For analysis of nuclear hnRNP K expression, specimens in which >50% of tumor cells displayed strong staining were defined as "high level of nuclear hnRNP K" and those where ≤50% of tumor cells stained strongly were defined as "low level of nuclear hnRNP K." For analysis of TP expression, specimens in which >25% of tumor cells displayed stronger staining than normal epithelium were defined as "high TP" and those where ≤25% of tumor cells stained strongly were defined as "low TP." TP-positive and hnRNP K–positive tumor cells in representative microscopic fields were scored independently by two experienced pathologists.

Cytotoxicity assay. The NPC-TW02 cell line (24) was maintained in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2 atmosphere. Induction of endogenous TP expression in NPC-TW02 cells was done using the treatment of serum deprivation. NPC-TW02 cell clone stably expressing ectopic TP were established by transfection with the TP expression plasmid, pcDNA3.1-TP (see Supplementary Data) followed by selection and maintenance with 500 μg/mL G418. For cytotoxicity assays, TW02 and TW02/TP cells were incubated with 5'-DFUR (Sigma-Aldrich). Following incubation for 72 h, plasma membrane externalization of phosphatidylserine was analyzed using the Vybrant Apoptosis Assay Kit (Invitrogen) with 340 (2 × 106) cells and PBS and adjusted to a concentration of 2 × 106/mL in binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl2 (pH 7.4)]. Alexa Fluor 488-Annexin V (5 μL) was added to 100 μL of the cell suspension and incubated for 15 min at room temperature. Samples were analyzed (10,000 events) using a FACSCalibur Flow Cytometer (Becton Dickinson).

Western blotting. Whole-cell lysates were prepared by suspending cells in NP-40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Igepal CA-630, 1 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 mmol/L phenylmethylsulfonyl fluoride] and incubating on ice for 30 min. Lysates were centrifuged at 12,000 × g at 4°C for 10 min to pellet cell debris and the supernatant was retained. Protein concentration in clarified lysates was determined using the Bradford reagent. Equal amounts of protein (30 μg) were resolved by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in Tris-Tween 20, membranes were incubated at room temperature for 2 h with primary anti-TP and anti-c-myc epitope antibodies (Santa Cruz Biotechnology) and anti-tubulin and anti-actin antibodies (MBIos). Membranes were subsequently incubated with secondary antibodies coupled to horseradish peroxidase and developed with ECL detection reagents (Amersham Pharmacia Biotech).
RNA interference. NPC-TW02 cells were transfected with 50 nmol/L dsRNA duplex and 50 μg dsRNA transfection reagents (TransIT-TKO) according to the manufacturer’s protocol (Mirus Bio). SMARTpool reagents containing a mixture of four 21-bp RNA duplexes targeting TP were purchased from Dharmacon; negative control small interfering RNA (siRNA) were synthesized by Research Biolabs (Singapore). Oligonucleotide sequences are presented in Supplementary Table S3. Twenty-four hours after transfection, the medium containing siRNA complexes was replaced with serum-free medium containing 5′-DFUR. After an additional 72 h in culture, cells were harvested and used in cytotoxicity assays and for Western blotting to confirm that the transfected RNA duplexes had the ability to knock down the expression of their respective targets.

Statistical analysis. All statistical analyses were done using the SPSS 13.0 statistical software package. Relationships between hnRNP K and TP expression and clinicopathologic characteristics were evaluated using the Pearson χ² test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards model was applied for multivariate analysis to determine the independence of each prognostic factor. The cutoff value used to define high hnRNP K and TP was based on the percentage of cytoplasmic hnRNP K and strong TP staining in tumor cells as determined from receiver operating characteristics curve analysis. In vitro data were analyzed with the Student’s t test. Differences were considered significant at a level of P < 0.05.

Results

hnRNP K and TP expression and correlation of hnRNP K with elevated TP in NPC. hnRNP K expression status in NPC has

<table>
<thead>
<tr>
<th>Table 1. Correlation of hnRNP K and TP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TP expression</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Cytoplasmic hnRNP K</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Nuclear hnRNP K</td>
</tr>
<tr>
<td>High</td>
</tr>
<tr>
<td>Low</td>
</tr>
</tbody>
</table>

*With statistic significance.
not been investigated. To determine the expression and cellular localization of hnRNP K in NPC tumors, we examined 121 NPC biopsies by immunohistochemical staining with hnRNP K antibody. As shown in Fig. 1, hnRNP K was primarily located in the nucleus of tumor cells; 39 samples (32% in Table 1) showed negative for cytoplasmic staining but positive for nuclear staining (Fig. 1A) and were defined as “cytoplasmic negative.” The remaining 82 samples (68% in Table 1) had both cytoplasmic and nuclear hnRNP K staining (Fig. 1B) and were defined as “cytoplasmic positive.” The same representative samples with lower magnification (×200) were shown in Supplementary Fig. S1. In contrast, hnRNP K was expressed weakly in normal nasopharyngeal epithelium and was exclusively nuclear (Fig. 1C). Because we have found that cytoplasmic hnRNP K is involved in enhancing TP mRNA stability,7 we further analyzed the correlation of hnRNP K and high TP in NPC tissues by immunohistochemistry using anti-TP antibody. As shown in Fig. 1D and E, 49 (40%) and 72 (60%) samples showed low and high levels of TP staining, respectively. In contrast, TP was expressed weakly in normal nasopharyngeal epithelium (Fig. 1F). A statistical analysis of NPC expression data using the Pearson χ2 test showed that both cytoplasmic and nuclear hnRNP K (see Supplementary Fig. S2) were positively correlated with TP expression (P < 0.001 and P = 0.025; Table 1). These

7 Unpublished data.
results also suggested that both hnRNP K and TP are highly expressed in NPC tumor cells.

**Patient characteristics and hnRNP K.** To evaluate the prognostic significance of cytoplasmic and nuclear hnRNP K under current therapeutic protocols, we subjected the same retrospective cohort of 121 NPC patients to the clinical outcome assessment study. Patient characteristics and clinical features are summarized in Supplementary Tables S1 and S2. The median age at diagnosis was 45 years (range, 22.0-78.2), with a male-to-female ratio of ~3:1. The clinicopathologic features were comparable between patient subgroups classified according to cytoplasmic and nuclear hnRNP K. No significant correlations were found between hnRNP K and other clinicopathologic features, including age, gender, tumor stage, node stage, American Joint Committee on Cancer stage, and chemotherapy.

**Association of cytoplasmic hnRNP K and elevated TP with OS.** Kaplan-Meier survival analysis was carried out to determine OS for patients as a function of cytoplasmic hnRNP K positivity. As shown in Fig. 2A, there was a significant difference in OS between patients with positive and negative staining for cytoplasmic hnRNP K ($P = 0.007$). Similarly, OS between patients grouped by high and low expression of TP, a regulatory target of hnRNP K, were significantly different ($P = 0.007$; Fig. 2B). In contrast, no statistical correlation between OS and nuclear hnRNP K were obtained ($P = 0.877$; Supplementary Fig. S3A). We next conducted a multivariate analysis of cytoplasmic hnRNP K or TP with age, gender, tumor stage, node stage, and chemotherapy. Our data indicated that cytoplasmic hnRNP K ($P = 0.020$) and high TP expression ($P = 0.010$) are independent prognostic predictors of poor OS (Tables 2 and 3).

**Nonassociation of cytoplasmic hnRNP K and elevated TP with DFS and LRFS.** Repeating the above analyses using DFS as the endpoint, we found no statistical correlation between DFS and cytoplasmic hnRNP K ($P = 0.835$; Fig. 2C), nuclear hnRNP K ($P = 0.646$; Supplementary Fig. S3B), or TP ($P = 0.318$; Fig. 2D) expression levels. Similarly, we found no association between LRFS and cytoplasmic hnRNP K ($P = 0.282$; Fig. 2E), nuclear hnRNP K ($P = 0.823$; Supplementary Fig. S3C), or TP ($P = 0.546$; Fig. 2F) expression levels.

**Association of cytoplasmic hnRNP K and elevated TP with DMFS.** Among the 121 patients, 26 developed distant metastases after the initial radiotherapy. Of these, 24 of 26 were cytoplasmic hnRNP K positive (92.3%) and 2 were negative (7.7%). All analyses were then repeated using the date of distant metastasis diagnosis as the endpoint. As shown in Fig. 2G, the presence of cytoplasmic hnRNP K was positively correlated with DMFS ($P = 0.003$) in NPC patients. When DMFS was related to TP expression levels, a significant association was found ($P = 0.001$; Fig. 2H). No statistical correlation between DMFS and nuclear hnRNP K was found ($P = 0.808$; Supplementary Fig. S3D).

**TP enhances drug sensitivity of NPC cells.** The 5-year survival rates under current standard therapies were 87.8% and 82.1% in NPC patients with low TP and absence of cytoplasmic hnRNP K, respectively. In contrast, high TP and positive staining for cytoplasmic hnRNP K in cancer cells were associated with shorter overall patient survival (48.6% and 56.1%; Fig. 2A and B). To assess the possibility of exploiting elevated TP expression therapeutically, we tested the TP dependence of sensitivity to the TP-targeting drug, 5’-DFUR, using TP-expressing NPC cells in conjunction with RNA silencing techniques. NPC-TW02/TP cells stably expressing

---

**Table 3. Multivariate analysis of the association between TP expression and OS of NPC patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients ($N = 121$), hazards ratio (95% confidence interval)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgeíasMedian</td>
<td>1.00 (Reference)</td>
<td>0.104</td>
</tr>
<tr>
<td>â‰‡Median</td>
<td>0.59 (0.308-1.116)</td>
<td>0.674</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.00 (Reference)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.86 (0.436-1.711)</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00 (Reference)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.35 (0.524-3.469)</td>
<td>0.535</td>
</tr>
<tr>
<td>3</td>
<td>2.35 (0.934-5.916)</td>
<td>0.069</td>
</tr>
<tr>
<td>4</td>
<td>4.29 (1.767-10.438)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Node stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.00 (Reference)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.67 (0.695-4.025)</td>
<td>0.251</td>
</tr>
<tr>
<td>2</td>
<td>2.21 (0.836-5.621)</td>
<td>0.110</td>
</tr>
<tr>
<td>3</td>
<td>2.32 (0.909-5.917)</td>
<td>0.078</td>
</tr>
<tr>
<td>4</td>
<td>0.44 (0.199-0.949)</td>
<td>0.010*</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.00 (Reference)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.29 (1.767-10.438)</td>
<td>0.001*</td>
</tr>
<tr>
<td>TP expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1.00 (Reference)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3.44 (1.351-8.746)</td>
<td></td>
</tr>
</tbody>
</table>

*With statistic significance. Median age is 45 y.
Fig. 3. TP enhances drug sensitivity of NPC cells. A, TP increases 5'-DFUR-induced apoptosis in NPC cells. NPC-TW02 cells stably expressing TP (NPC-TW02/TP) cultured with serum and NPC-TW02 cells cultured with or without serum (to induce TP expression) were treated with 5'-DFUR (10 or 30 μmol/L). Cytotoxicity was measured by Annexin V staining after incubating with drug for 72 h. TP levels were determined by Western blotting using anti-TP or anti-Myc antibodies. B, TP siRNA reduced 5'-DFUR-induced apoptosis. Twenty-four hours after transfecting NPC-TW02 cells with a control siRNA or TP siRNA, cells were serum starved and treated with 30 or 100 μmol/L 5'-DFUR. Cytotoxicity was assessed by determining the fraction of apoptotic cells by Annexin V staining 72 h later. Mean ± SD from at least three experiments. *, P < 0.05.
expression enhanced the sensitivity of NPC cells to the capecitabine intermediate prodrug, 5’-DFUR. The implication is that NPC patients with cytoplasmic hnRNP K and/or high TP may selectively benefit from capecitabine therapy. The upstream modulator of TP, hnRNPK, may also be attractive as a candidate target molecule in the development of future cancer therapies. In summary, we showed a significant association of cytoplasmic hnRNP K with elevated TP expression in tumor samples from NPC patients. Both cytoplasmic hnRNP K and high TP expression may be independent prognostic markers for OS and DMFS in NPC patients. In addition, elevated TP expression enhanced the sensitivity of NPC cells to the capecitabine-intermediate prodrug, 5’-DFUR, presenting an opportunity for future therapeutic development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Heterogeneous Ribonucleoprotein K and Thymidine Phosphorylase Are Independent Prognostic and Therapeutic Heterogeneous Ribonucleoprotein K and Thymidine Phosphorylase Are Independent Prognostic and Therapeutic Markers for Nasopharyngeal Carcinoma

Lih-Chyang Chen, Chuen Hsueh, Ngan-Ming Tsang, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/12/3807

Cited articles
This article cites 25 articles, 5 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/12/3807.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/14/12/3807.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.