PTK2 Promotes Uveal Melanoma Metastasis by Activating Epithelial-to-Mesenchymal Transition

Zhongyi Fan

The Third People's Hospital of Shenzhen

Jingjing Duan

Tianjin Medical University

Lei Zhang

Xuanwu Hospital Capital Medical University

Qiong Chen

The Third People's Hospital of Shenzhen

Wen Xiao

The Third People's Hospital of Shenzhen

Pu Luo

The Third People's Hospital of Shenzhen

Ling Shao

The Third People's Hospital of Shenzhen

Jingye Meng

The Third People's Hospital of Shenzhen

Jianghong An

The Third People's Hospital of Shenzhen

Gengwei Zhang

The Third People's Hospital of Shenzhen

Xiaohua Tan (✉ xiaohua_t@126.com)

The Third People's Hospital of Shenzhen <https://orcid.org/0000-0002-1392-534X>

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Abstract

Background: Uveal melanoma (UM) is an aggressive primary intraocular tumor in adults, with high metastatic capacity and high morbidity. However, the mechanisms of UM metastasis have not been clearly elucidated.

Methods: The PTK2 expression and activation were performed in the Cancer Genome Atlas (TCGA) database and 25 patients of UM. The role of PTK2 in promoting metastasis was explored in vitro and in vivo. Subsequently, we revealed the correlation between PTK2 expression and epithelial-to-mesenchymal transition (EMT). Finally, we explored the reason for the high expression of PTK2 in UM.

Results: Our study found that protein tyrosine kinase 2 (PTK2) was overexpressed in UM specimens, and as a novel independent risk factor, its overexpression predicted the poor survival of UM patients. For the molecular mechanism, PTK2 promoted EMT phenotype, thus leading to tumor metastasis in UM cells. Subsequently, we have demonstrated that PTK2 was a functional gene of chromosome 8q gain accounting for UM metastasis, providing a novel molecular mechanism for the aberrantly expression and activation of PTK2 in UM.

Conclusion: Our data reveal the important role and mechanism of PTK2 in the metastatic process of UM, which may clue to a new predictive biomarker for UM metastasis and a new therapeutic target for UM treatment.

Introduction

Uveal melanoma (UM) remains the most common primary intraocular malignancy in adults. Approximately 85% of ocular melanomas occur in the uvea, of which about 90% of UM cases involve the choroid, while the rest are limited to the iris or ciliary body(1–5). Primary UM can be treated by surgery or radiotherapy, and the local recurrence rate is low. However, due to the high metastatic potential of UM, up to 50% of UM patients will develop distant metastatic diseases, usually to the liver, lung, bone or other organs(3, 5–8). In order to further improve the prognosis of UM patients, a variety of treatment schemes including transpupillary thermotherapy, photocoagulation, charged particle irradiation and immunotherapy have been explored in the past decade. However, without effective treatments for metastatic UM, most UM patients have a survival time of less than 12 months after a diagnosis of metastases(9–13). Therefore, it is necessary to further investigate the mechanism of UM metastasis, identifying biomarkers that can predict metastasis in advance and finding new therapeutic targets for metastatic UM.

Revealing the molecular mechanism in the process of UM metastasis remains the main challenge of current UM research. Protein tyrosine kinase 2 (PTK2), also known as focal adhesion kinase (FAK), is a non-receptor protein tyrosine kinase, which can mediate integrin and growth factor signaling pathways(14–16), thus regulating a variety of cell functions including cell survival, proliferation, adhesion and migration(3, 14–20). Many studies have shown that the expression and activity of PTK2

are up-regulated in multiple tumors and related to poor prognosis(14, 18, 20–23). PTK2 was also reported to be overexpressed in UM specimens (24, 25), and correlated with metastases of UM (25, 26). However, the regulatory mechanism of PTK2 overexpression in UM remains largely unclear, and its role in the metastatic process of UM has not been clarified yet.

In this study, we confirmed that PTK2 was up-regulated in UM samples and correlated with the poor survival of UM patients. PTK2 expression, which was enhanced by chromosome 8q gain, stimulated UM metastasis by activating the EMT process both *in vitro* and *in vivo*. Our data reveal novel roles of PTK2 in the metastatic process of UM, suggesting that PTK2 may be used as a predictive biomarker for UM metastasis and a novel therapeutic target for UM therapy.

Methods

Clinical sample and IHC. Our study collected 25 paraffin-embedded, archived UM tumor samples from Xuanwu Hospital between 2010 and 2016, with the approval of the Institutional Review Committees of Xuanwu Hospital. Informed consent was obtained from all patients. All specimens were histologically confirmed as UM, without previous chemotherapy or radiotherapy and prior history of other malignancies. For IHC, the samples were deparaffinized with dimethylbenzene, rehydrated with gradient ethanol and then treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Microwave treatment was used to retrieve the antibody-binding epitopes of antigens, and the specimens were subsequently incubated with 10% normal serum to reduce nonspecific binding. The samples were incubated with the primary antibodies, such as rabbit anti-PTK2 (1:100, ab40794, Abcam, Cambridge, MA, USA) and rabbit anti-phospho-PTK2 (Tyr397) (1:50, 44-624G, Invitrogen, Carlsbad, CA, USA), for 1h at room temperature. Then biotinylated anti-rabbit secondary antibody was added. 3,3'-diaminobenzidine was used as chromogenic agent and hematoxylin was used for counterstaining. Finally, the score was calculated by multiplying the intensity of the staining (low, 1+; medium, 2+; strong, 3+) by the percentage of stained cells (0–100%).

Cell lines and culture. Human Uveal melanoma cell lines (OCM-1 and MUM-2B) were purchased from American Type Culture Collection (ATCC), and mycoplasma contamination was excluded. Routinely, UM cells were cultured in DMEM medium (Invitrogen) or RPMI 1640 medium (Invitrogen) supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% FBS (HyClone, Logan, UT, USA).

Lentiviruses and reagents. Lentiviruses carried PTK2 shRNA vector were purchased from Genescript (Wuhan, China). Cell lines with PTK2 stable knock-down were selected in $1\mu\text{g ml}^{-1}$ puromycin for 2 months. And pooled clones were screened using standard immunoblot protocols.

Gene set enrichment analysis. GSEA was performed using GSEA 2.2.3 software. PTK2 expression was regarded as a numerical variable. We applied the continuous CLS file of PTK2 spectrum to the phenotypic tag in GSEA. 'Pearson' was used for ranking genes and the other parameters were set to their default values.

Western blot. Western blot analysis was conducted according to the standard procedure. Both anti-PTK2 (Abcam) and anti-phospho-PTK2 (Tyr397) (Invitrogen) was used at dilutions of 1:500. Anti-GAPDH (1:2,000, Sigma) was used as a loading control.

RNA extraction and RT-PCR. According to the manufacturer's protocol, total mRNA was extracted with RNA Extraction Kit (Takara Bio Inc., Shiga, Japan). For mRNAs detection, RNA was firstly reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara Bio Inc.), and the real-time PCR was then performed with SYBR Premix Ex Taq™ (Takara Bio Inc.). Primers were listed in Supplementary Table 1.

Cell migration and invasion assays. Cell migration ability was determined using wound healing assays. When UM cells grown in 6-well plates attached as confluent monolayers, a 1-ml pipette tip was used to create the wound. Then, cells were washed with PBS and cultured in the medium with 1% FBS for 24 hours to allow the wound healing. For transwell invasion assay, chambers (Corning Inc.) were coated with Matrigel (BD Biosciences) on the upper surface. Equal cells were seeded into the upper chambers and cultured with medium containing 0.1% FBS, while cell medium containing 20% FBS was placed in the lower well as a chemoattractant. After 24 hours, cells that successfully invaded through the Matrigel were fixed with 4% paraformaldehyde and then stained with crystal violet before taking photographs.

Tumor xenografts in vivo. Animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Xuanwu Hospital. BALB/c nu/nu mice (6-week-old, male) were purchased and randomly divided into different groups (5 mice per group). 1×10^6 MUM-2B cells with PTK2 knockdown were injected intravenously through the lateral tail vein of NOD-SCID mice (6-week-old, male, 6 mice per group) to establish the metastasis model. And all mice were maintained for 50 days until the analyses by the micro-PET.

PET imaging of glucose uptake in mice. In our study, the PET imaging of mice was conducted using an animal PET scanner (Philips Corp.) according to indications. Briefly, mice were anesthetized with pentobarbital followed by the intravenous injection with 3.7 MBq (100 μ Ci) of ^{18}F radio-labeled fluorodeoxyglucose (^{18}FDG). To obtain attenuation correction data, a 5-minute emission scan was performed in the prone position at 60 minutes after injection, and a 10-minute delay scan was then acquired at 2 hours after injection. The tumor radioactivity of each mouse was calibrated against a known aliquot of the injected tracer and presented as the percentage of the injected dose of tissue.

Statistical Analysis. All statistical analyses were performed using SPSS 22.0. All data were representative of at least three independent experiments and illustrated as the means $\pm$ standard deviation (SD). The Student's t test was used for two-group comparisons. Survival curves according to PTK2 expression were estimated with the Kaplan-Meier method, and the log-rank test was used to assess significance. The univariate and multivariate analyses of risk factors were performed with Cox Regression analysis or Logistic Regression analysis. All statistical tests were two-sided. In all assays, a P value < 0.05 was considered statistically significant.

Results

PTK2 is over-expressed and activated in UM samples

To investigate the PTK2 status in UM samples, the cBioPortal program (<http://www.cbioportal.org/>) was used to screen for the PTK2 expression level of UM patients in the Cancer Genome Atlas (TCGA) database. We found that PTK2 expression was altered in 46 (46/80, 57.5%) UM cases. Among them, 45 (45/46, 97.8%) cases had the overexpression of PTK2 due to amplification or mRNA upregulation (Fig. 1A). We then assessed total PTK2 level and its activated forms phospho-PTK2 (Y397) expression between UM cancer tissues and normal uveal by immunohistochemical staining analysis. The results showed that PTK2 staining and phospho-PTK2 (Y397) expression was only slightly detectable in 25 normal uveal tissues, whereas both the two forms were markedly overexpressed in UM cancer tissues (Fig. 1B and Fig. 1C). Further analyses of TCGA UM dataset indicated that patients with higher PTK2 expression had significantly shorter overall survival (OS) than that of lower PTK2 levels (Fig. 1D). Meanwhile, univariate Cox analysis also showed that the overexpression of PTK2 was related to an increased risk of death in TCGA UM cases ($P = 0.002$). We then put the factors whose P value ≤ 0.1 in the univariate analysis into the subsequent multivariate Cox regression analysis. After adjustment for patient age and tumor basal diameter, the multivariate Cox analysis also demonstrated that the PTK2 level was a negative prognostic factor for OS in UM patients, independent of the above risk factors (hazard ratio (HR), 3.633; 95% CI, 1.415–9.328; $P = 0.007$) (Table 1). These data indicated that PTK2 overexpression and activation, which was frequent in UM, predicted adverse prognosis of UM. Therefore, further investigation is warranted to verify the prognostic value of PTK2 expression in UM patients.

PTK2 increases UM metastasis in vivo and in vitro

As the dominant cause of treatment failure, the occurrence of distant organ metastasis often leads to the death of UM patients. To investigate whether PTK2 could affect the metastasis process in UM cells, we firstly examined the effects of PTK2 on the metastasis ability by up-regulating PTK2 or silencing PTK2 (PTK2 knockdown or activity inhibition) in UM cells. Both the wound-healing assay and the transwell invasion experiments showed that the overexpression of PTK2 enhanced the migration and invasive capacity of UM cells (Fig. 2A-2C). Conversely, we observed the opposite effects in PTK2-silenced cells (Fig. 2A-2C, and Supplementary Figure 1A and 1B). The mRNA levels of PTK2 were significantly upregulated in PTK2-overexpressing UM cells, and reduced in PTK2 knockdown cells accordingly (Fig. 2D). The levels of phospho-PTK2, the activation form of PTK2, changed by the overexpression or knockdown of PTK2. The inhibition of PTK2 activity was detected with immunofluorescence assay. As shown in Figure 2E, PTK2-overexpressing cells exhibited higher fluorescence intensity of phospho-PTK2 level, whereas PTK2-silenced cells observed lower fluorescence intensity, especially after treatment with PTK2 inhibitors, compared to their control cells (Fig. 2E).

Moreover, PET imaging of the pulmonary metastasis mice models revealed that the number of tumor foci in the lungs and their radioactivity levels were much lower in the PTK2-knockdown group than that of the control group (Fig. 2F). It was showed that the number of nodules spread throughout the pulmonary

region in the resected lungs was clearly declined in the PTK2-knockdown group compared to the controls (Fig. 2F). The above results strongly support the promoting role of PTK2 in the metastasis process of UM.

PTK2 enhances EMT to promote UM metastasis

To examine the mechanism by which the metastasis ability of UM cells was up-regulated during the overexpression of PTK2, we analyzed the potential metastasis-related signalling pathways influenced by the differential expression of PTK2. The GSEA results indicated that PTK2 expression was positively correlated with metastasis, also positively correlated with epithelial-to-mesenchymal transition (EMT) (Fig. 3A and Supplementary Figure 2). Since EMT is well known to be the key mechanism involving in tumor metastasis, and the role of PTK2 in the EMT has been verified in various kinds of cancer cells, we hypothesized that PTK2 may promote UM cells metastasis through EMT. As expected, the overexpression of PTK2 decreased the levels of E-cadherin, regarded as the epithelial marker, whereas increased the mesenchymal markers such as N-cadherin, Slug, Snail and Vimentin in OCM-1 cells (Fig. 3B). And the opposite results were observed after inhibiting PTK2 activity (Fig. 3C), suggesting that PTK2 could induce EMT. Moreover, we found that PTK2 overexpression could enhance the morphologic changes of UM cells from a polarized epithelial phenotype to an elongated fibroblastoid phenotype, and PTK2-silenced cells indicated the opposite phenomenon (Fig. 3D). These results suggest that PTK2 may increase UM cells metastasis through the regulation of EMT.

PTK2 is a function gene in the chromosome 8q gain accounting for UM metastasis

To identify potential mechanism underlying the PTK2 aberrant expression in UM, we focused on the location of *PTK2* gene. Following the analysis of the NCBI database, we found that the sequence of PTK2 gene is located in chromosome 8q24.3 (Fig. 4A). Previous results exactly showed that aberrant chromosome 8q gain was associated with UM metastasis and prognosis. Therefore, the overexpression of PTK2 may result from chromosome 8q gain. Based on the metastasis status of TCGA UM data, we divided these patients (n=80) into two groups (54 patients without metastasis, and 26 patients with metastasis). Then the PTK2 expression were compared between the two groups. Metastatic tumors tended to have higher PTK2 expression ($P=1.7\times10^{-3}$) (Fig. 4B). Moreover, four factors including PTK2 high expression, BAP1 mutation, the gain of chromosome 8q and the loss of chromosome 3 (the last three factors have been confirmed as UM metastasis risk factors in previous studies [25-27]), were all associated with metastasis status for UM; however, only the high expression of PTK2 was significantly related to UM metastasis after the multivariate Logistic regression analysis, with a hazard ratio of 3.69 (95% CI, 1.25-10.9; $P=0.018$) (Fig. 4C). These results suggest that PTK2, as a function gene located in chromosome 8q, play dominant role in the chromosome 8q gain which is essential for UM metastasis.

Discussion

Surgery and radiotherapy are effective treatments that effectively prevented the local recurrence in UM (3, 5–8). However, distant metastasis has always been a very troublesome clinical problem, indicating that it

will be of great clinical benefit to elaborate the detailed molecular mechanisms underlying the metastasis of UM. PTK2 play an essential role in the cell migration, and has been recognized as a positive regulator of metastasis in various cancer types (16, 17, 20, 25, 26). Here, we found that PTK2 promotes EMT phenotype, leads to tumor metastasis in UM cells. In addition, as a novel independent risk factor, PTK2 overexpression predicts a poor prognosis of UM patients. Based on the results, we support the notion that PTK2 is an important contributor to metastasis in UM tissues, suggesting that PTK2 inhibitors may control the metastasis and progression of UM. By far, the efficacy of PTK2 inhibitors (i.e. GSK2256098, VS-6063 or Defactinib) have been explored in several phase I or II clinical trials in various advanced malignant tumors (<https://clinicaltrials.gov/>) (27–29). The data we presented is supportive for further translational research in the clinical application of PTK2 inhibitors in UM.

As a tyrosine kinase, unlike BRAF and EGFR mutations in melanoma or lung cancer (30, 31), PTK2 exists no mutations contributing to PTK activation in UM patients (<http://www.cbioportal.org>). Thus, it is of great benefit to fully investigate the biological basis for PTK2 over-activation, facilitating the future development of novel therapeutic targeted drugs. Recently, Faingold *et al.* found that Hsp90 maybe a regulator for PTK2 expression and activation (24). Gangemi *et al.* demonstrated that Mda-9/syntenin could promote the cell motility and invasion capacity through connecting surface integrin signals to PTK2 activity (26). Here, we verified that the upregulated PTK2 mRNA was the main cause for PTK2 overexpression, which was frequently accompanied by its high activity. We indicated that PTK2 is located in chromosome 8q24.3. Previous studies have confirmed that aberrant chromosome 8q gain was frequent in UM and contributed to UM metastasis (32–34). Our results also demonstrated that the expression of PTK2 were higher in metastatic cases of UM. Logistic regression analyses indicated that the high expression of PTK2 as well as chromosome 8q gain, BAP1 mutation, chromosome 3 loss were significant predictors for UM metastasis in the univariate analyses, consistent with previous results in other studies. However, only the overexpression of PTK2 was the independent risk factor after the multivariate analyses. These data provide a new molecular mechanism to predict UM metastasis, indicating PTK2 inhibitors may be used as potential therapeutic agents for metastatic UM patients with PTK2 overactivation.

In many malignant tumors, EMT is very important for tumor cells to obtain more advanced capacities of local invasion and distant metastases. PTK2 signalling contributes to this EMT change (20). We found that PTK2-overexpressing UM cells performed high expression of Slug, and Snail, acting as stimulators in EMT change reported by Asnaghi *et al.* (35). More importantly, PTK2 also enhanced mesenchymal phenotype, promoted the invasion and migration abilities of UM cells and accelerated tumor metastasis in mice xenograft models. This provides a new molecular mechanism for PTK2-mediated UM cell metastasis, predicting that PTK2 inhibitors may be another potential therapeutic drug for metastatic UM patients with PTK2 overactivation.

Conclusions

To conclude, we found that PTK2 is overexpressed in UM specimens and identified as a novel independent risk factor. Through the promotion EMT phenotype, PTK2 overexpression led to tumour metastasis in UM. Subsequently, we have identified that PTK2 as a functional gene of chromosome 8q gain, providing a new layer of molecular mechanism for the aberrantly expression and activation of PTK2 in UM. Our study not only provides novel insight into molecular mechanisms underlying UM metastasis, but also indicates that PTK2 may be a biomarker for susceptibility to UM and presented the supportive data for further translational research in potentially expanded PTK2 inhibitors indication in UM.

Declarations

Ethics approval and consent to participate Clinical sample and animal experiments implemented in this study were approved by the Ethic Committee of the Xuanwu Hospital.

Consent for publication: Not applicable.

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors have no conflict of interest.

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Authors' contributions: X. Tan, G. Zhang, Z. Fan and J Duan conceived the project, analyzed the data and wrote the manuscript. J. An, L. Zhang, J. Meng and Q. Chen, designed and performed the experiments. W. Xiao, and P. Luo, performed some of the experiments. L. Shao performed the animal experiments and analyzed the clinical samples. All Authors have seen and approved the manuscript being submitted.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure1

Figure 1

PTK2 is overexpressed and activated in UM. (A) Genetic alteration of PTK2 analyzed by the cBioPortal program for UM cases of TCGA database. (B and C) PTK2 was upregulated (B) and activated (C) in 25 UM specimens (T) compared to paired adjacent non-cancerous tissues (N) according to IHC scores. Representative immunohistochemical staining were displayed in the right images. Scale bar, 200µm or

50µm as indicated. (D) Kaplan-Meier estimates of 80 UM patients with available OS data in the TCGA UM patients. P value < 0.05 was considered as statistically significant.

Figure2

Figure 2

PTK2 enhances the metastasis of UM in vitro and in vivo. (A) Wound healing assays for PTK2-overexpressed and PTK2-silenced OCM-1 cells compared to the corresponding control cells. (B and C) Representative images and quantification of cell invasion using a Matrigel invasion chamber. Invasive

cells were fixed and stained with crystal violet. (D) PTK2 expression was detected by RT-PCR. (*P < 0.01). (E) PTK2 activation was detected by immunofluorescence staining analyses. (F) Representative PET images of the living mice injected with stable PTK2-knockdown MUM-2B cells or negative control cells. Images and radioactivity detection of resected lungs showed that PTK2 knockdown clearly inhibited the number of the nodules spread throughout the pulmonary region. Data are shown as mean ± SD (n = 6). P value < 0.05 was considered as statistically significant.

Figure3

Figure 3

PTK2 promotes EMT of UM cells. (A) GSEA analyses showed that PTK2 expression was positively correlated with metastasis gene signatures and also positively correlated to epithelial-to-mesenchymal transition gene signatures in the TCGA UM dataset. (B and C) RT-PCR analysis of the expression levels of EMT markers in PTK2-overexpressed (B) and PTK2-silenced (C) cells compared to the corresponding control cells (*P < 0.05). (D) Morphologic changes in the indicated cells are shown in the photographs.

Figure4

A

Location: 8q24.3 See PTK2 in [Genome Data Viewer](#)
Exon count: 47

Annotation release	Status	Assembly	Chr	Location
109.20210226	current	GRCh38.p13 (GCF_000001405.39)	8	NC_000008.11 (140657900..141002079, complement)
105.20201022	previous assembly	GRCh37.p13 (GCF_000001405.25)	8	NC_000008.10 (141667999..142012178, complement)

B

C

Figure 4

PTK2 upregulation in UM is due to chromosome 8q gain. (A) Location of PTK2 gene from NCBI database. (B) Comparison of the expression levels of PTK2 in patients with metastasis or without metastasis. (C) Forest plot showed the HR of PTK2, chromosome 8q gain, BAP1 mutation and chromosome 3 loss contributed to UM metastasis. P value < 0.05 was considered as statistically significant.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xlsx](#)
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