TMTP1, a Novel Tumor-Homing Peptide Specifically Targeting Metastasis

Wanhua Yang, Danfeng Luo, Shixuan Wang, Rui Wang, Rui Chen, Yan Liu, Tao Zhu, Xiangyi Ma, Ronghua Liu, Gang Xu, Li Meng, Yunping Lu, Jianfeng Zhou, and Ding Ma

Abstract

**Purpose:** Tumor metastasis continues to be the major obstacle to cancer therapy and the leading cause of cancer-related death. Methods used to detect metastasis, especially occult metastases, have received a great deal of attention. In this study, a novel selective peptide was assessed for its specific binding to metastasis.

**Methods:** The FliTrx bacterial peptide display system, an alternative to phage peptide display, was used to identify a 5-amino acid peptide termed TMTP1 (NVVRQ), which binds to the highly metastatic prostate cancer cell line PC-3M-1E8. The synthetic TMTP1 was tested in vitro for its binding specificity and affinity to highly metastatic cancer cells. The tumor targeting assays were done in vivo by i.v. injection of FITC-conjugated TMTP1 into tumor-bearing mice.

**Results:** TMTP1 specifically bound to a series of highly metastatic tumor cells, including prostate cancer PC-3M-1E8, breast cancer MDA-MB-435S, lung cancer PG-BE1, and gastric cancer MKN-45SCI, in vitro and in vivo but not to the poorly metastatic or nonmetastatic cell line, including prostate cancer PC-3M-2B4, breast cancer MCF-7, lung cancer PG-LH7, or murine fibroblast cell NIH/3T3. FITC-TMTP1 strongly and specifically targeted the metastasis foci in tumor-bearing mice 24 h after i.v. peptide injection. Moreover, the occult metastases were specifically detected by FITC-TMTP1.

**Conclusion:** Our results suggest that TMTP1 is a potential strategy for the development of new diagnostic tracers or alternative anticancer agents for tumor metastasis.

Metastasis is responsible for most therapeutic failures in cancer treatment and leads to death in most cancer patients. Once cancer is diagnosed, it is important to know whether the disease is confined to the primary site or has spread either regionally or systemically (1). At initial diagnosis, approximately 70% of cancer patients can be cured surgical removal of tumors. Adjuvant radiation therapy and/or chemotherapy are beneficial for cancer patients postoperation (2–5). However, a proportion of patients with no evident systemic dissemination will develop recurrent disease or metastasis after “curative” therapy (6, 7). In these cases, the cancer has clearly undergone occult systemic spread and is undetectable by routine methods including careful clinical, pathologic, biochemical, and radiologic evaluation. Sometimes, the metastatic cancer cells form microfoci (<2 mm) and maintain a balance between proliferation and apoptosis without evoking clinical symptoms (8–11). Evasion of the host immune system by these tumor cells leads to systemic spread to tissues and eventually ruin the host (5, 12–14). The 5-year survival rate in patients with recurrence or metastasis is <50% (15, 16). Although many new treatment modalities have been developed, they seldom improve the survival rate of patients with cancer metastasis (17–20).

Diagnostic approaches for micrometastasis are essential to controlling metastasis. Heterogeneous tumor cells can be divided into clones with highly or poorly metastatic potential. Highly metastatic cancer cells, which are prone to spreading to other tissues and forming metastases, express unique molecular markers that distinguish them from cancer cells with poorly metastatic or nonmetastatic potential. These markers are potentially useful as diagnostic markers and therapeutic targets (21–25). However, it is a major obstruction to detect such specific markers of metastasis. Peptide display technology has been used to study protein-protein interaction for over 20 years. The strength of this technology is the ability to identify interactive proteins and other molecules without prior knowledge of the nature of the interaction (26). The identified “homing” peptides are promising alternatives to currently used biomolecules for targeting metastatic cells because of their rapid blood clearance, increased diffusion and tissue penetration, nonimmunogenic nature, and ease of synthesis (27–31).
In the present study, we used the bacterial peptide display system, FliTrx, an alternative to phage peptide display, to identify a new peptide that binds to highly metastatic cancer cells. In the FliTrx system (Invitrogen), a phagemid vector (pFliTrx) allows the display of peptides directly on the surface of *Escherichia coli* by using two proteins: the major bacterial flagellar protein (FlIC) and thioredoxin (TrxA). Peptide libraries are cloned in frame within the active site loop of thioredoxin, which is inserted into the dispensable region of the FlIC gene. The resulting fusion protein assembles into the flagella protruding from the bacterial cell surface. The dodecamer peptide library is constrained by a disulfide bridge, which results in a stable conformation for the synthetic peptide, and is resistant to degradation (32, 33).

**In vitro** screening of the FliTrx library with the highly metastatic prostate cancer cell line PC-3M-1E8 derived a 5-amino acid peptide, NVVRQ (termed TMTP1). We carefully assessed the accuracy and specificity of TMTP1 binding to a series of cell lines of various metastatic potential and its targeting to the metastatic foci.

**Materials and Methods**

**Cell lines.** The paired highly metastatic and nonmetastatic human prostate cancer cell lines PC-3M-1E8 and PC-3M-2B4 and human lung cancer cell lines PG-BE1 and PG-LH7, as shown in Supplementary Table S1 (34), were kindly provided by Dr. Jie Zheng (Beijing University; refs. 35, 36). The human breast cancer cell lines MDA-MB-435S, MDA-MB-231, and MCF-7 and the murine fibroblast cell line NIH/3T3 were obtained from the American Type Culture Collection. All other cultured cell lines and amino acid sequence of TMTP1 are shown in Table S1 (34), were kindly provided by Dr. Jinjun Li (Shanghai Cancer Institute, Medical College of Shanghai Jiao Tong University; refs. 37, 38). Fresh tumor fragments were obtained from the SLAC Laboratory Animal Co. and injected s.c. into the posterior trunk with a 5-0 absorbent suture. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed with 1-0 surgical monofilament.

**Construction of mouse models.** Four-week-old BALB/c nu/nu mice were obtained from the SLAC Laboratory Animal Co. and injected i.p. into the posterior trunk with a single dose of 2 × 10^6 tumor cells to induce tumor (PC-3M-1E8, PC-3M-2B4, and MDA-MB-435S cell lines). Three weeks after tumor cell injection, the mice were euthanized to harvest the tumor fragments. Fresh tumor fragments (~ 2 mm^3) were then implanted s.c. into the posterior trunk of anesthetized mice.

The mouse model of MKN-45sci orthotopic gastric cancer, which has liver-specific metastasis potential, was kindly provided by Dr. Jinjun Li (Shanghai Cancer Institute, Medical College of Shanghai Jiao Tong University; refs. 37, 38). Fresh tumor fragments were obtained as described above. The stomach of the anesthetized mouse was exposed, and part of the serosal membrane was scraped with a forceps. One 1-mm^3 tumor piece was fixed on the scraped site of the serosal surface with a 5-0 absorbent suture. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed with 1-0 sutures.

The mouse model of MDA-MB-435S orthotopic breast cancer was induced by injection of a single dose of 2 × 10^6 tumor cells to the mammary fat pad.

**Peptide library screening and FliTrx clones binding.** A random 12-mer cyclic peptide display library, FliTrx (Invitrogen), was screened. Tumor-targeting FliTrx clones were isolated from the FliTrx library by combined in vitro screening according to the manufacturer’s protocol. Quantification of binding selectivity was determined by counting the specific binding in a series of tumor cell lines. Fluorescein imaging revealed that TMTP1 bound to all highly metastatic tumor cells tested, including prostate cancer PC-3M-1E8, breast cancer MDA-MB-435S, lung cancer PC-BE1, and gastric cancer MKN-45sci (Fig. 1E-I). By contrast, TMTP1 did not bind to the poorly metastatic or nonmetastatic tumor cells, including prostate cancer PC-3M-2B4, breast cancer MCF-7, lung cancer PC-LH7, or murine fibroblast NIH/3T3.

**Results**

**Identification of a peptide that specifically binds to a highly metastatic prostate cancer cell line.** Targeting peptides were selected in vitro by screening of the FliTrx library with the highly metastatic prostate carcinoma cell line PC-3M-1E8 for four rounds. For each round, 1 × 10^8 FliTrx cells were added to a cell culture dish with the nonmetastatic human prostate cancer cell line, PC-3M-2B4, for negative selection, and then incubated for 1 h with PC-3M-1E8 cells for positive selection. After four rounds, 100 individual FliTrx clones were selected and their encoding inserts were sequenced and analyzed for potential repetitive peptide motifs. One of the peptide sequences enriched in the selected clone pool was NVVRQ, which we named TMTP1. The computer-generated mimic models and amino acid sequence of TMTP1 are shown in Fig. 1A to C. An in vitro binding experiment showed that TMTP1-displaying FliTrx clone 27 or 50 specifically bound to PC-3M-1E8 cells 12.6 or 11.7 times over control PC-3M-2B4 cells, respectively (Fig. 1D). A synthetic TMTP1 with the sequence GCGXXXXXGC (XXXXX = variable residue, C = cysteine, and G = glycine), which is inserted into the dispensable region of the FlIC gene, was used in the competitive inhibition assays and completely inhibited specific binding of the clones to PC-3M-1E8 cells (Fig. 1D).

**TMTP1 specifically binds to highly metastatic tumor cells in vitro.** To determine whether TMTP1 peptide binds to highly metastatic tumor cells in vitro, FITC-conjugated TMTP1 was used to determine the specific binding in a series of tumor cell lines. Fluorescein imaging revealed that TMTP1 bound to all highly metastatic tumor cells tested, including prostate cancer PC-3M-1E8, breast cancer MDA-MB-435S, lung cancer PC-BE1, and gastric cancer MKN-45sci (Fig. 1E-I). By contrast, TMTP1 did not bind to the poorly metastatic or nonmetastatic tumor cells, including prostate cancer PC-3M-2B4, breast cancer MCF-7, lung cancer PC-LH7, or murine fibroblast NIH/3T3.

1 http://www.ncbi.nih.gov/BLAST
cells (Fig. 1I-L). svTMTP1, whose amino acid composition resembles that of TMTP1, was synthesized as a control to identify the specific binding characteristics of TMTP1. FITC-conjugated svTMTP1 showed no positive binding to any highly metastatic tumor cells (Fig. 1M-P). Flow cytometric analysis further confirmed the specific binding of TMTP1 to highly metastatic PC-3M-1E8 and MDA-MB-435S tumor cells (Fig. 1Q and R). Similarly, the lower E-cadherin expression levels were detected by flow cytometry in both PC-3M-1E8 and MDA-MB-435S cells compared with the paired poorly metastatic PC-3M-2B4 and MCF-7 cells (Fig. 1Q and R).

TMTP1 binding location to tumor cells was time dependent. Confocal images showed that FITC-TMTP1 was bound to PC-3M-1E8 cell membranes 10 min after incubation (Fig. 2A) and was gradually internalized into the cytoplasm after 30 min (Fig. 2B). After 6 h incubation, strong fluorescein staining was detected in the cytoplasm, and fluorescein-labeled peptide was partially apparent in the nucleoplasm (Fig. 2C). No positive staining was detected in PC-3M-2B4 cells (Fig. 2D).

**TMTP1 targets highly metastatic tumors in vivo.** The initial in vitro experiments showed that TMTP1 specifically bound to highly metastatic tumor cells. We next sought to determine whether TMTP1 could target highly metastatic tumor cells in vivo. For this purpose, three highly metastatic tumor cell lines, PC-3M-1E8, MDA-MB-435S, and MKN-45sci, and one nonmetastatic tumor cell line, PC-3M-2B4, were used to induce s.c. tumors (PC-3M-1E8 and PC-3M-2B4) or orthotopic tumors (MDA-MB-435S and MKN-45sci) in BALB/c nude mice. Mice with tumors 1 to 1.5 cm\(^3\) in size were used...
for the in vivo targeting studies. FITC-TMTP1 (210 μg) was injected via the tail vein and allowed to circulate for 24 h. Tumors and control organs were then excised and processed for frozen sectioning. As shown in Fig. 3, 24 h after i.v. injection, TMTP1 specifically targeted all three highly metastatic tumors, including PC-3M-1E8 (Fig. 3A), MDA-MB-435S (Fig. 3B), and MKN-45sci (Fig. 3C). The fluorescence of TMTP1 appeared mainly in the cell cytoplasm (Fig. 3C, inset). By contrast, no TMTP1 fluorescence was detected in non-metastatic PC-3M-2B4 tumor (Fig. 3D). Furthermore, animals injected with control peptide FITC-svTMTP1 had no detectable positive fluorescence in the PC-3M-1E8 (Fig. 3E) or MDA-MB-435S tumors (Fig. 3F).

To determine the distribution of TMTP1 in tumor-bearing mice, organs and tissues were removed, frozen sectioned, and examined for fluorescence 24 h after i.v. injection of FITC-TMTP1. Specific fluorescence conjugation was detected in PC-3M-1E8 xenografted tumor tissue (Fig. 4A). However, no

---

**Fig. 2.** Internalization and nuclear translocation of TMTP1 by tumor cells. PC-3M-1E8 cells were incubated with 1 μmol/L FITC-conjugated TMTP1 from 10 min to 6 h. Cell nuclei were counterstained with 500 μg/μL propidium iodide. The fluorescence in the tumor cell was examined by Olympus Fluoview FV1000 CLSM. A, PC-3M-1E8 cells incubated with FITC-TMTP1 for 10 min. B, tumor cells incubated with FITC-TMTP1 for 30 min. C, tumor cells incubated with FITC-TMTP1 for 6 h. D, PC-3M-1E8 cells incubated FITC-svTMTP1 for 6 h. Magnification, ×600. C, inset, magnification, ×1,800.
specific fluorescence was detected in any normal organs and tissues (Fig. 4B-I). Weak fluorescence was observed in kidney tubuli presumably as a result of uptake of free FITC-TMTP1 from the glomerular filtrate (Fig. 4F). The homing assay with FITC-svTMTP1 showed no obvious fluorescence staining in PC-3M-1E8 xenografted tumor tissue or normal tissues (Fig. 4J). In PC-3M-1E8 xenografted tumor tissue, the fluorescence intensity of FITC-svTMTP1 was 23.5-fold lower than that of FITC-TMTP1 (Fig. 4J).

**TMTP1 targets tumor metastases.** The data above showed specific binding of TMTP1 to highly metastatic tumor cells in vitro and in vivo. An unresolved question was whether TMTP1 could target metastatic foci, particularly occult micrometastases in the host. This possibility was investigated by using two metastatic mouse models: BALB/c nu/nu mice with lymph node metastases after s.c. inoculation with prostate cancer PC-3M-1E8 cells (Fig. 5A) and mice with mesentery lymph node metastases (Fig. 5B) or liver metastases after orthotopic implantation of gastric cancer MKN-45sci cells, multiple gray nodules were visible on the capsular surface of the liver. They typically appeared as nodules 0.5 to 1.5 mm in size, which were centrally umbilicated (Fig. 5E). They contained necrotic plaques with heavy lymph cell infiltration and infrequent pyknotic neoplastic cells inside (Fig. 5F). These foci were examined by three independent pathologists and were not determined to be typical metastatic lesions in morphology. Further immunohistochemical staining with anti-CA72-4 antibody, a specific marker to gastric tumor, showed positive staining of the neoplastic cells in these foci (Supplementary Fig. S3). The ability of FITC-TMTP1 to specifically bind to these microfoci (Fig. 6G) was unexpected. In our later examination, 21 to 28 days after tumor implantation, we saw typical metastatic lesions (Fig. 6H), which were confirmed by pathologists (Fig. 6I). FITC-TMTP1 accurately targeted these metastatic tumors (Fig. 6J). These findings show that TMTP1 has the remarkable capability to specifically target metastatic foci, particularly the very early stage of metastatic foci that could only be identified by specific immunohistochemical staining.

**Similarity searching of human proteins for sequences containing the TMTP1 core.** We searched SWISSPROT for human proteins containing the TMTP1 peptide core sequence (NVVRQ). Only four human proteins exactly matched the sequence, Ran-binding protein 6, pannexin-2, Ran-binding protein 5, and zinc finger MYM-type protein 3. Both Ran-binding proteins 6 and 5 have conserved armadillo/protein 5, and zinc finger MYM-type protein 3. Both Ran-binding proteins 6 and 5 have conserved armadillo/h -catenin-like repeat domains, which have been implicated in protein-protein interactions. Pannexin-2 belongs to the innexin family, which forms the structural components of gap junctions. Zinc finger MYM-type protein 3 contains a metallochaperone-like domain and is associated with cancer-causing chromosomal translocations. When we extended the searching range to include single positive substitution patterns such as [NDHS]VVRQ or N[VILM]VRQ, we obtained more than 10,000 hits but could not deduce any common characteristics.

**In vivo targeting assay.** FITC-conjugated TMTP1 (210 µg) or svTMTP1 was injected via the tail vein of mice and allowed to circulate for 24 h. Subsequently, the mice were perfused with PBS through the left ventricle to remove blood and unbound peptides. Tumors were then excised, and frozen sections were prepared to examine fluorescence in xenograft tumors, including PC-3M-1E8 (A), MDA-MB-435S (B), MKN-45sci (C), and PC-3M-2B4 (D) after TMTP1 injection, or in PC-3M-1E8 (E) and MDA-MB-435S (F) after svTMTP1 injection. Nuclei were counterstained with 4',6-diamidine-2-phenylindole (blue). Magnification, × 200. C inset, ×1,200.
Discussion

Metastasis recurrence is considered to be the result of free tumor cells or micrometastases already present in secondary sites when the primary neoplasm is removed (8–11). The course of disease in most patients with metastasis is difficult to reverse (12). Therefore, methods used to detect and eliminate occult metastases in cancer patients have received much attention. Several molecular markers have been evaluated for their ability to detect micrometastases (21, 25, 28, 43–46).

Highly metastatic cancer cells, which are capable of aggressive invasion and stable resistance to chemotherapy, express unique molecular markers or lose some molecules such as E-cadherin, which is involved in the control of cell migration and invasion (41, 47). In this study, we screened a Flitrx library with the highly metastatic prostate cancer cell line PC-3M-1E8 in vitro to select peptides that home to cancer cells and their metastases.

The identified TMTP1 peptide, which contains the core sequence NVVRQ, was designed and synthesized with the structure GCGNVVRQGC. Glycine residues provide a spacer function, permitting the physical formation of a loop structure, and the flanking cysteines allow for the formation of a disulfide loop, thus constraining and mimicking the binding conditions of the initial selection process. TMTP1 specifically bound to highly metastatic tumor cells including prostate cancer PC-3M-1E8, breast cancer MDA-MB-435S, lung cancer PG-BE1, and gastric cancer MKN-45 in vitro and in vivo. However, it did not bind to the poorly metastatic or nonmetastatic cell lines PC-3M-2B4, MCF-7, and PG-LH7 or the murine fibroblast cell line NIH/3T3. This indicates that TMTP1 possesses excellent targeting or homing properties for metastatic tumors. In vitro and in vivo binding experiments with the control peptide svTMTP1, whose amino acid composition resembles that of TMTP1, further showed the specific targeting property of TMTP1.

Fig. 4. Distribution of TMTP1 in PC-3M-1E8 tumor-bearing mice. The targeting assay was done as mentioned in Materials and Methods. The distribution of FITC-conjugated TMTP1 in dissected tumor (A) and organs, including heart (B), liver (C), spleen (D), lung (E), kidney (F), brain (G), prostate (H), and intestine (I), were examined by fluorescence microscopy (Nikon TE1000-S). Magnification, ×100. The fluorescence intensity was quantified by using Image-Pro Plus 5.1 (Media Cybernetics, J). The autofluorescence background in mice that did not receive fluorescent compound was subtracted from the experimental values. svTMTP1 was used as the control peptide. Columns, average of three independent experiments; bars, SD.
Occult metastases or micrometastases, by definition, are not detected by even the most sophisticated imaging techniques such as positron emission tomography. Some of these occult foci can remain dormant without any signs and symptoms for years (8, 10). Even if dormant, these occult metastases maintain the potential to proliferate and recur. When the balance tips toward malignant proliferation, malignant cells progressively corrode tissues and organs till they thoroughly wreck the host. TMTP1 specifically targeted celiac lymph node metastases of PC-3M-1E8 tumors, the seeding foci, and the mesentery lymph node metastases of MKN-45sci tumors. Remarkably, occult foci even less than 1 mm in diameter was specifically targeted. TMTP1 also specifically recognized atypical liver micrometastases that contained infrequent neoplastic cells. It is conceivable that some neoplastic cells from parts of these lesions may ultimately escape the immune surveillance of the host and become clinical metastases (48). Our study shows that TMTP1 has a remarkable ability to target very early stage of occult metastasis foci. This could significantly manage metastasis or recurrence by detecting and eradicating concealed tumor cells before they evade the host’s immune surveillance and cause damage to the host.

The remarkable tumor-homing efficiency of TMTP1 may be due to its propensity to be internalized by cells. Cells that bind TMTP1 transport it across the cell membrane and into the cytoplasm and nucleoplasm. In this regard, TMTP1 is similar to the Tat peptide and other cell-penetrating peptides, which are also taken up by cells (42, 49). An important difference is that TMTP1 peptides are cell specific, targeting the highly metastatic cells that display the “receptor” for this peptide. Peptide internalization is likely to contribute to its effectiveness in becoming concentrated in the targeted tumors. We also observed that incubating PC-3M-1E8 cells with TMTP1 resulted in an obvious dose-dependent inhibition of cell viability and induced apoptosis of tumor cells.2

Ongoing experiments are in progress to determine the mechanism in which TMTP1 induces apoptosis. In the meantime, we have prepared TMTP1-sTRAIL and TMTP1-DT fusion proteins, in which human soluble tumor necrosis factor-related apoptosis-inducing ligand or diphtheria toxin is fused with the TMTP1 peptide. The antitumor activities of these fusion proteins were evaluated in vitro and in vivo and have revealed promising anticancer activity.3

It is important to identify the receptor that TMTP1 binds at the cell surface, which will help us to better understand the molecular properties of TMTP1. A BLAST search with the TMTP1 core sequence revealed four proteins, which do not contribute to metastasis, and the search for single positive substitutions yielded redundant proteins with no common biological characteristics. We are now working to isolate the TMTP1 receptor. Affinity chromatography (ProFound Pull-Down) and time-of-flight delayed extraction MALDI mass spectrometer (Bruker Autoflex) are being used for this purpose. The preliminary results of mass spectrometry hinted the aminopeptidase P, a GPI-linked membrane dipeptidase

---

2 W. Yang, et al. unpublished findings.
3 R. Liu, et al. unpublished findings.
expressed on the surface of vascular endothelial cells and lymphoid cells, may be involved. However, this result needs to be further confirmed.

TMTP1 promises to be a useful tool for detecting occult metastases. Furthermore, eradication of occult micrometastases by coupling radioactive isotopes or anticancer agents to the TMTP1 peptide would be greatly beneficial in protecting cancer patients from metastasis or recurrence.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interests.

Acknowledgments

We thank Drs. Ping Wu, Ping Gong, Jianhui Wen, and Hongxing Tang and Yunqin Chen of Hunan University for fluorescence quantification support and Dr. Luo Qinmin for whole-body imaging.

References

14. Chambers AF, Naumov GN, Varghese HJ, Nadkarni KV, MacDonald IC, Groom AC. Critical steps in...
Clinical Cancer Research

TMTP1, a Novel Tumor-Homing Peptide Specifically Targeting Metastasis

Wanhua Yang, Danfeng Luo, Shixuan Wang, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/17/5494

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2008/09/03/14.17.5494.DC1

Cited articles
This article cites 49 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/17/5494.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/14/17/5494.full#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, use this link
http://clincancerres.aacrjournals.org/content/14/17/5494.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.