B16 melanoma cells in order to develop "chemo-thermo-immuno (CTI) therapy". The feasibility of this strategy is tested in a pilot preliminary clinical study with a limited number of patients.

Experimental Approach and Results: The therapeutic protocol of NPrCAP/magnetite (M) against the primary transplanted tumor with or without AMF once a day every other day for a total of three treatments not only inhibited the growth of primary transplant, but also prevented the growth of the secondary, re-challenge transplant and increased life span of the host mice. HSP production at the site of primary transplant and $CD8^{+}$T cell infiltration at the site of the re-challenge melanoma transplant were seen. The *in vivo* study using re-challenged B16 F1, F10 and OVA melanoma cells after NPrCAP with/without M treatment showed that NPrCAP alone can suppress the transplanted secondary tumor through melanoma-specific host immune response through CD8 T cells as the effecter. Specifically "first B16 melanoma transplanted" mice treated by *ip* administration of NPrCAP for 3-5times showed not only growth inhibition of primary transplant but also the marked and significant growth inhibition of "second re-challenge melanoma".

Our preliminary clinical trials showed that NPrCAP/polyethylene glycol (PEG)/dextran magnetite (DNM) administration into the *in situ* melanoma nodules with AMF exposure can suppress distant metastatic melanoma lesions and increased the life span of advanced melanoma patients.

Significance: Our study indicates that NPrCAP combined with magnetite nanoparticles with AMF exposure has strong chemo-and immunotherapeutic properties for melanoma targeting CTI therapy strategy. In addition, it was found that NPrCAP alone could be a good candidate for melanoma adjuvant therapy.

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MELANOMA: NOVEL, RESEARCH-BASED THERAPY; Utilization of melanogenesis substrate, NPrCAP for exploiting melanoma-targeting drug and its conjugation with magnetite nanoparticles for melanoma chemo-thermo-immunotherapy

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BACKGROUND: Exploitation of a specific biological property is one of the best approaches for developing novel cancer-targeted drugs. This study shows how we can translate the basic research of melanin pigmentation to developing a novel nanomedicine for better management of advanced-stage melanoma patients, to whom we can currently offer very limited treatments.

RATIONALE AND APPROACH: Melanogenesis substrate, N-propionyl cysteaminylphenol (NPrCAP: amine analog of tyrosine) may provide a unique drug delivery system (DDS) because of its selective incorporation into melanoma cells. It may also act as a melanoma-targeted therapeutic drug because of its production of highly reactive free radicals (melanoma-targeted chemotherapy). Utilization of magnetite nanoparticles can also be a good platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to the alternating magnetic field (AMF).

RESULTS: We examined the feasibility of this approach in experimental study using *in vivo* and *in vitro* B16 melanoma cells and preliminary clinical study with a small number of advanced melanoma patients. Four types of drugs were synthesized by conjugating NPrCAP with magnetite nanoparticles (M) and two of them, i.e., NPrCAP/M and NPrCAP/PEG/M were used for animal and human studies respectively. The therapeutic protocol against the primarily transplanted tumor with or without AMF once a day every other day for a total of three treatments not only inhibited the growth of primary transplant, but also prevented the growth of the secondary, re-challenge transplant and increased life span of the host mice. HSP70 production at the site of primary transplant and $CD8^+$T cell infiltration at the site of the re-challenge melanoma transplant were seen. The inhibition of re-challenge melanoma transplantation was seen in almost of all of the cases with AMF exposure. Importantly, however, the treatment of NPrCAP/M without AMF or NPrCAP alone also showed a significant number of cases, approximately 50%, with the growth inhibition of the second re-challenge melanoma transplantation. Four patients entered in the preliminary clinical trial by following the basic outline of this animal protocol and two of them showed PR and CR.

CONCLUSION: In this study we are able to establish a novel CTI therapy which is based upon the combination of (1) direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effect of melanogenesis-targeted drug and (2) indirect killing by immune reaction (*in situ* vaccination) after exposure to AMF. Our results indicate the successful development of a strategy that a tumor-specific drug delivery system to melanoma cells is achieved by selective incorporation of NPrCAP/magnetite nanoparticle conjugates and selective cell death can be achieved by not only NPrCAP itself but also exposure to heat, which then can induce HSP expression through either necrotic or non-necrotic process or combination of the two, without damaging non-cancerous tissues and establish immune reaction targeted to other metastatic melanoma lesions.

SIGNIFICANCE This study shows the feasibility of translational approach from the basic research of melanin pigmentation to the development of novel CTI therapy by establishing not only melanoma-targeted chemo-thermotherapy but also *in situ* vaccination immunotherapy to advanced melanoma through exploitation of melanogenesis cascade.

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メラノーマ形質を分子標的としたナノメデシン化学・温熱・免疫療法の基礎と臨床

転移性メラノーマに対し、メラニン形成を分子標的とした新規温熱療法である「化学・温熱・免疫療法;chemo-thermo-immunotherapoy (CTI 療法)」確立の基礎と臨床を紹介する。
我々はメラノーマの新規癌治療法の開発に際して、個々の癌腫に特異的な形質発現を標的として利用し、治療効果のある成分を特異的形質代謝系に積極的に取り込ませ、癌組織を選択的に破壊し、さらにそれを介して最後の癌治療法といわれている生体内産生癌ペプチド療法の開発を目的として研究を行っている。殊にメラニン形成酵素、チロシナーゼの特異的基質であるチロシンのアミン誘導体(NPrCAP, NAcCAP)を合成した。NPrCAP, NAcCAP はメラノーマ細胞に選択的に取り込まれ、チロシナーゼと反応し細胞障害性ラジカル(酸化ストレス)を産生し、メラノーマの増殖抑制を示すが、この選択的化学療法効果を増加させるために微細鉄粒子表面に NPrCAP を重合させた薬剤(NPrCAP/M)を合成し、その後交換磁場照射により温熱を発生させ選択的温熱細胞殺効果を起こさせ、結果として生じる熱ショック蛋白(HSP)とメラノーマペプチド結合体を介した温熱免疫療法により、遠隔転移メラノーマの消滅を図った。
本研究では、デリバリー性と高拡散性を有した新規ナノ微粒子結合体の合成に成功し、新たなメラノジェネシスを分子標的とする次世代型新規薬剤の開発が可能となった。本 CTI 療法で誘導されるメラノーマ特異的 CTL の解析に関しては現在 CTL の質的、または量的変化を確認しており、さらに今後の臨床的応用への道が期待されている。

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Refinement of chemo-thermo-immunotherapy using magnetite nanoparticles conjugated with NPrCAP for the treatment of malignant melanoma

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We have already shown that melanogenesis substrate, N-propionyl-4-S-cysteaminylphenol (NPrCAP) is specifically taken up by melanoma cells and inhibits their growth by producing cytotoxic free radicals. By taking advantage of this unique chemical agent, we have established melanoma targeting intracellular hyperthermia by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/M) upon exposure to alternating magnetic fields (AMF). NPrCAP/M with AMF to C57BL mice with B16 melanoma inhibited the growth of re-challenged melanoma cells inoculated on to the opposite side of the body after the removal of the initially inoculated melanoma in mice. It also provided thermal heat which resulted in the melanoma necrosis and immune reactions generating cytotoxic T cells and regressed distant skin metastases in human clinical trials.
However, when this particle (NPrCAP/M), was injected to the melanoma cells, it aggregated around the cells. We needed to refine the particle to be incorporated more into each cell. We successfully made new particles (NPrCAP/PEG/DNM) in which maleimide-PEG5000-Carboxyl-NHS is used to combine NPrCAP and dextran magnetite. The particle size of the new one is 50~60nm, which is smaller that the previous one (350~500nm). That does not aggregate around the tumor cells, and was more diffusely distributed in the melanoma cells inoculated on the mice and showed growth inhibition more effectively than the previous particle. Novel chemo-thermo-immunotherapy with this new particle can be developed for melanoma patients by exploiting melanogenesis cascade.

Successful introduction of melanoma targeted, *in situ* peptide vaccine through chemo-thermotherapy by conjugation of melanogenesis substrate, NPrCAP, with magnetite nanoparticles

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The management of advanced-stage melanoma patients is still a difficult challenge for both clinicians and scientists. This study is to introduce melanoma-targeted, *in situ* vaccine therapy by using peptides introduced in the melanoma lesions through chemo-thermotherapy.

In this approach melanogenesis substrates were exploited and N-propionyl cysteaminylphenol (NPrCAP) was synthesized to develop a unique melanoma-targeted therapeutic drug because of its selective incorporation into melanoma cells and production of highly reactive free radicals, which result in apoptotic cell death and melanoma antigen production, possibly through generation of heat shock protein (HSP) upon exposure to tyrosinase. In addition, magnetite nanoparticles were conjugated with NPrCAP (NPrCAP/PEG/M) to introduce thermotherapy because of non-apoptotic cell death and HSP generation upon exposure to alternating magnetic field (AMF).

We examined the feasibility of this "*in situ* chemo-thermotherapy introduced peptide (vaccine) for melanoma immunotherapy" by experimental study using *in vivo* and *in vitro* B16 melanoma cells and preliminary clinical study to advanced melanoma patients.

We found (1) that a tumor-specific drug delivery and cell death were achieved by NPrCAP itself and exposure to AMF, (2) that NPrCAP/M with AMF (and without AMF to lesser extent) not only inhibited the growth of primary transplant, but also prevented the growth of the secondary, re-challenge transplant and increased life span of the host mice, (3) that HSP70 production at the site of primary transplant and $CD8^+$T cell infiltration at MHC I expressing sites of the re-challenge melanoma transplant were seen, (4) that prevention of re-challenge transplant was not seen in those mice with pre-administration of anti-CD8 antibody and finally (5) that our preliminary clinical trial to stage III/IV patients showed PR and CR. $CD8^+$ T cells were seen predominantly at NPrCAP/PEG/M sites.

In conclusion, our study is the first report indicating that conjugation of melanogenesis substrate, NPrCAP, an amine derivative of sulfur homologue of tyrosine, with magnetite nanoparticles, can successfully be utilized to develop the *in situ* vaccine therapy, which is generated by chemo-thermotherapy, and effectively applicable to advanced-stage melanoma patients.