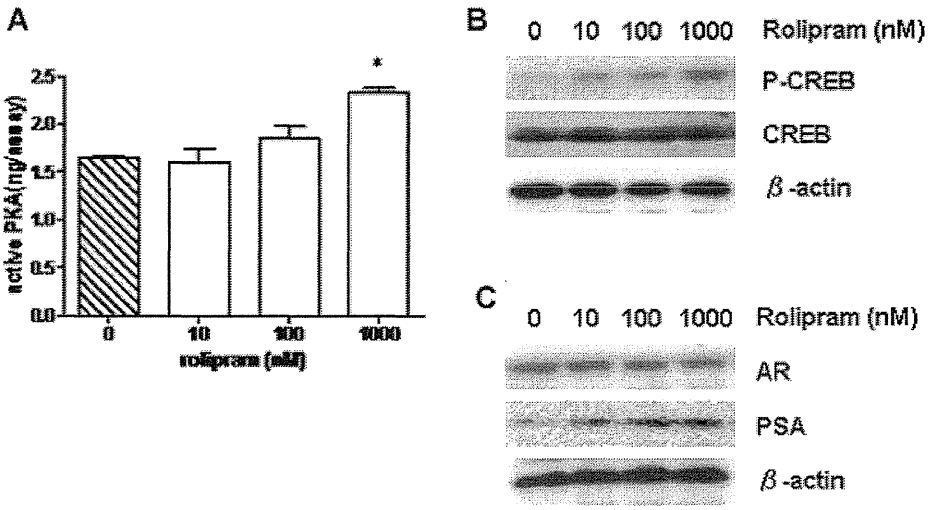

Fig. 3. PDE4B inhibitor, rolipram activates the PKA pathway and increases CREB phosphorylation. **A**: LNCaP cells were cultured with rolipram for 48 hr, and cell lysates were used for PKA kinase assay. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with LNCaP cells). **B**,**C**: Same lysates were analyzed for phosphoCREB (B), CREB (B), AR (C), and PSA (C) by SDS–PAGE and western blotting with specific antibodies. β-actin was used as a loading control.

activated in CxR and HPR50 cells. Next, we tried to uncover whether a PKA inhibitor could be an effective drug for CRPC. As shown in Figure 7B, H89 suppressed the cell growth of LNCaP as well as CxR and HPR50 cells.

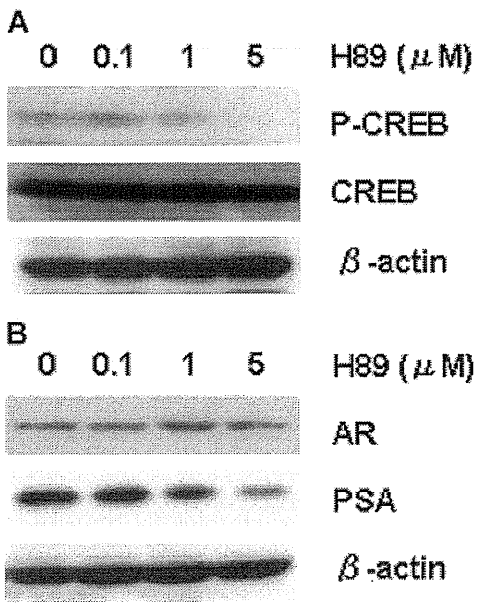

Fig. 4. PKA inhibitor, H89 decreases CREB phosphorylation and PSA, but not AR. **A**,**B**: LNCaP cells were cultured with H89 for 48 hr, and cell lysates (30 μg) were analyzed for phosphoCREB (A), CREB (A), AR (B), and PSA (B) by SDS–PAGE and Western blotting with specific antibodies. β-actin was used as a loading control.

PDE4B Expression Is Related to Prostate Cancer Progression

Finally, to examine the relationship between stage of prostate cancer and PDE4B expression, we analyzed PDE4B expression in prostate cancer tissues by quantitative real-time RT-PCR, using prostate cancer TissueScan Real-Time qPCR Arrays (OriGene). This array contains 9 normal prostate tissues and 39 prostate cancer tissues representing different clinical stages. As seen in the left section of Figure 7C, PDE4B expression was not different between a normal and cancerous prostate ($P = 0.3483$). However, surprisingly, in invasive and metastatic prostate cancer (stage III and IV), PDE4B was downregulated compared with stage II ($P = 0.0154$). These observations suggest that PKA activation by PDE4B downregulation must be important for prostate cancer to be more aggressive.

DISCUSSION

Feldman categorized five potential mechanisms by which CRPC can develop [22], including: (1) hypersensitivity of AR under very low level of androgens (the hypersensitive pathway), (2) decreasing the specificity of ligand binding and inappropriate activation of AR by various non-androgen steroids and androgen antagonists (the promiscuous pathway), (3) ligand independent activation of AR by phosphorylation (the outlaw pathway), (4) parallel or alternative survival pathways (the bypass pathway), and (5) malignant epithelial stem cells (the lurker cell pathway).

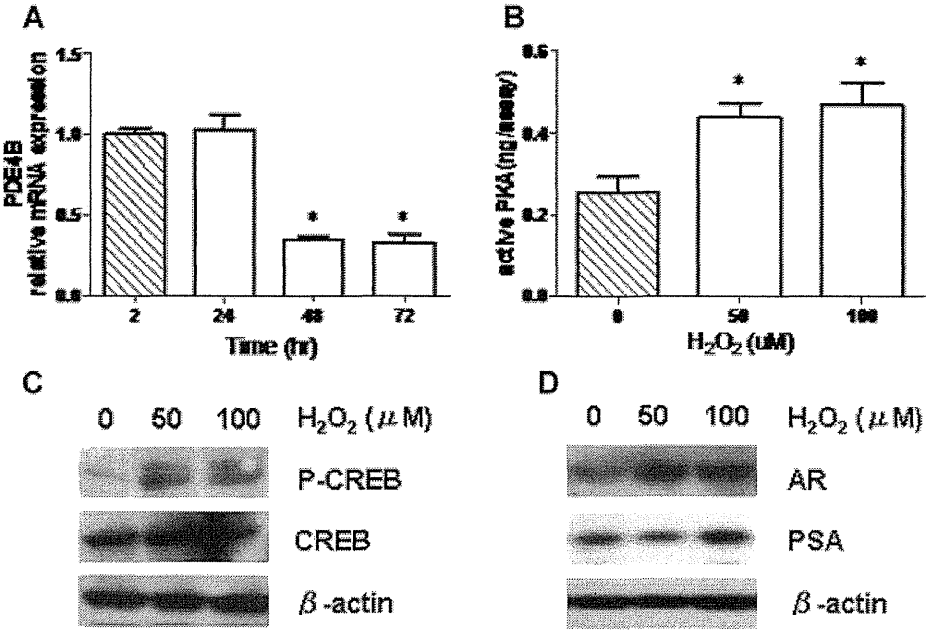

Fig. 5. Oxidative stress decreases PDE4B transcript expression level and activates the PKA pathway in LNCaP cells. **A**: LNCaP cells were cultured with 50 μM of hydrogen peroxide for 2 hr in serum free medium, which was refreshed. At the indicated times after the addition of hydrogen peroxide, the cells were harvested. Quantitative real-time PCR was performed. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^{*}P < 0.05$ (compared with LNCaP cells at 2 hr). **B**: LNCaP cells were cultured with the indicated concentration of hydrogen peroxide for 2 hr, and the medium was refreshed. At 48 hr after the addition of hydrogen peroxide, the cells were harvested. PKA kinase assay was also performed. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^{*}P < 0.05$ (compared with LNCaP cells). **C**,**D**: Using the same lysates (30 μg), western blot analysis was performed against phosphoCREB (C), CREB (C), AR (D), and PSA (D). β-actin was used as a loading control.

As PKA may modulate AR function by phosphorylation [14,23], it primarily falls into the outlaw pathway. While the PKA activator did not increase AR protein level, it did manage to enhance the AR activity by increasing AR-DNA binding [24]. Thus, it can be surmised that PKA activation enhances AR transactivation [3]. Consistent with the previous findings, our results showed that the modulations of PKA activity by PDE4B inhibition affected AR transactivation, but not AR expression. Furthermore, augmented AR activity confers castration resistance to androgen-dependent prostate cancer. Therefore, once PKA is activated by PDE4B downregulation, LNCaP cells can proliferate in an androgen-deprived condition. Furthermore, activation of PKA also induces tumor invasion and metastasis [25–27]. Our qPCR arrays data (Fig. 7C) indicate that in prostate cancer cells, PDE4B could be downregulated to activate PKA pathway and may contribute to tumor progression. The cAMP-specific PDE4 have four subfamilies (PDE4A, 4B, 4C, 4D) and there are multiple splicing variants of each gene [28]. PDE4s have multiple functions in intracellular signaling. These functions include the regulation of receptor desensitization and G protein switching as well as PKA and extracellular signal-regulated kinase activation [29]. PDE4s have been linked to a wide range of diseases including chronic obtrusive pulmonary disease (COPD), asthma, Parkinson's disease, schizophrenia, depression, and also cancer [30,31]. PDE4 promotes the progression of many kinds of tumor cell lines including brain tumor, osteosarcoma, and malignant melanoma [32–34]. PDE4D is overexpressed in human prostate cancer patient samples and knockdown of PDE4D has been shown to reduce the proliferation of prostate cancer in vivo and in vitro [35]. Analysis of our human samples of qPCR arrays suggests that PDE4B does not significantly increased in prostate cancer samples, and PDE4B knockdown in LNCaP cells promote proliferation in androgen deprived condition. Thus, PDE4 subtypes may have different functions in prostate cancer progression.

A PDE4 inhibitor is currently recommended as a candidate anticancer drug [36]. PDE4 inhibition activates mitochondrial apoptotic pathway and causes cell cycle arrest in hematological malignancies [36,37]. However, our data clearly showed that rolipram, a PDE4 inhibitor, did not suppress LNCaP nor the

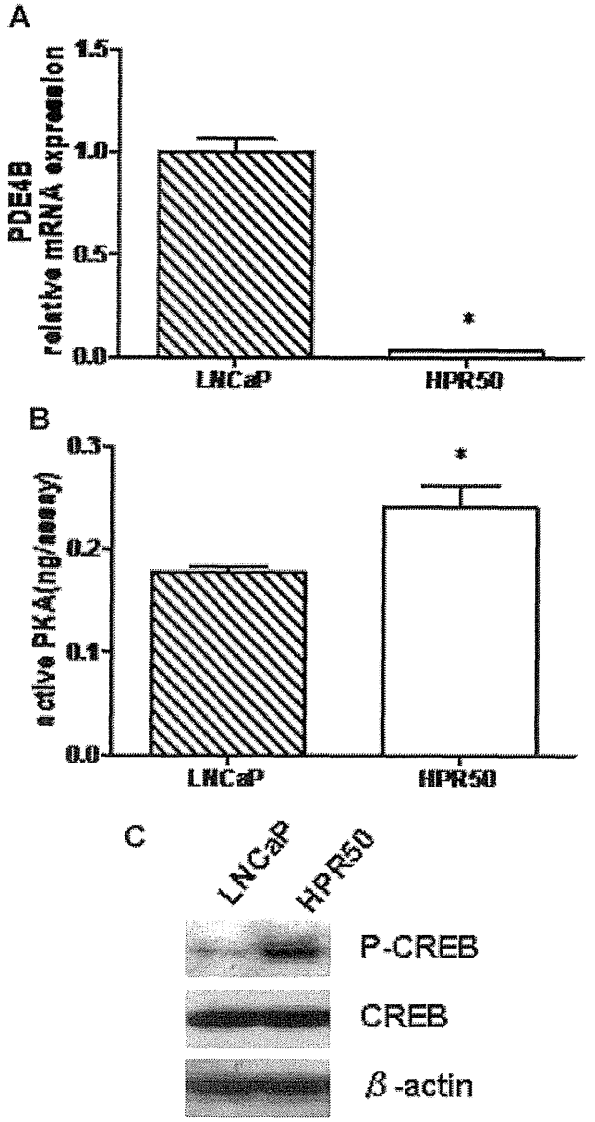

Fig. 6. PDE4B expression is downregulated but PKA activity is more activated in hydrogen peroxide-resistant LNCaP derivatives (HPR50 cells) compared with LNCaP cells. **A**: mRNA levels of PDE4B in LNCaP and HPR50 cells were analyzed by quantitative real-time PCR. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with LNCaP cells). **B**: Both LNCaP and HPR50 cells were cultured in charcoal-stripped medium for 48 hr, and a PKA kinase assay was performed. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with that of LNCaP cells). **C**: Whole cell extracts of LNCaP and HPR50 cells were analyzed for phospohoCREB and CREB by SDS–PAGE and Western blotting with specific antibodies. β-actin was used as a loading control.

proliferation of other prostate cancer cell lines even at concentrations of >50 μM (data not shown). Since rolipram potentiates androgen-induced AR-responsible activity in LNCaP cells [15], PDE4B

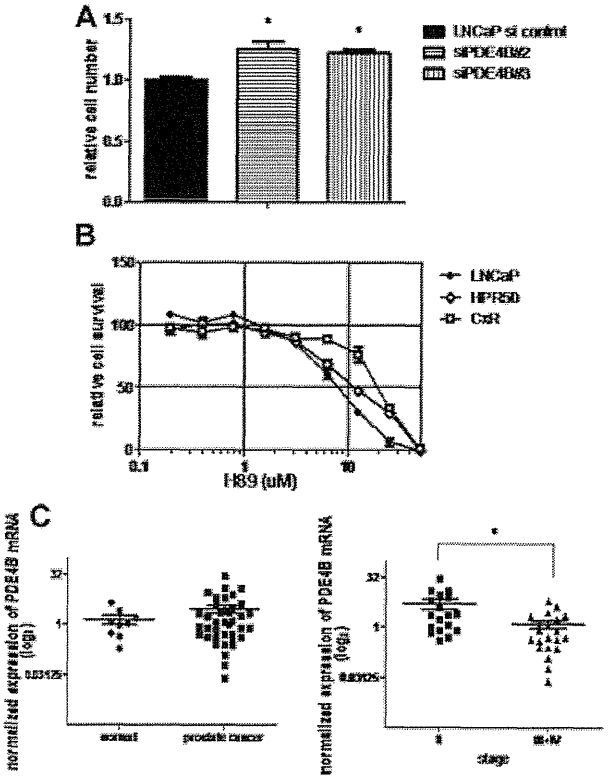

Fig. 7. PDE4B and PKA pathway regulate prostate cancer progression. **A**: LNCaP cells were transfected with 40 nM of control siRNA, PDE4B siRNA #2, or PDE4B siRNA #3, and cultured in a charcoal-stripped medium. After 96 hr, the cell numbers were counted. The relative number of LNCaP cells transfected with control siRNA was set as 1. All values are representative of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with LNCaP cells transfected with control siRNA). **B**: LNCaP, CxR, and HPR50 cells (2×10^3) were seeded into 96-well plates. The following day, various concentrations of H89 were applied in medium. After 48 hr, the surviving cells were stained with the Alamar Blue assay. Cell survival in the absence of drugs corresponded to 100%. All values represent the mean of at least three independent experiments. **C**: PDE4B expression in normal prostate and prostate cancer cDNAs was determined. The $\log_2$ of normalized (to β-actin) expression values relative to the mean expression of the normal is presented in normal prostate versus prostate cancer (left) or stage II versus stage III and IV prostate cancer (right). The line in the middle represents the median. Bars, ±s.d. $^*P < 0.05$ (compared with stage II).

inhibition appears to contribute to survival pathways in LNCaP cells. In other words, PDE4 related pathways may have different function among tumor types. Neuroendocrine (NE) cells have been observed in prostate cancers, and some clinical studies have proposed prostatic NE cells could be used as prognostic markers for tumor progression [38]. Increased cAMP was able to induce NE differentiation in both androgen dependent and independent cells [39,40].

Furthermore, NE differentiation was also induced by activated PKA [41]. NE differentiation is thought to be closely implicated in the development of castration-resistant or androgen-independent prostate cancer. Our finding of decreased PDE4B and enhanced PKA activity in CRPC cells seems compatible with this hypothesis.

Recent studies have reported an association between prostate cancer risk and oxidative stress [42,43]. Maintenance of an appropriate intracellular reactive oxygen species (ROS) level is important in keeping the redox balance and maintaining proper cellular signaling [44,45]. Furthermore, oxidative stress confers castration-resistant phenotype to LNCaP cells, suggesting that oxidative stress plays a key role in the progression to CRPC [17]. In this report, we found that oxidative stress by hydrogen peroxide reduced the expression PDE4B, resulting in an activation of the PKA pathway and acquiring the CRPC phenotype (Fig. 8). However, the mechanism of PDE4B downregulation by oxidative stress remains unknown. We previously reported that oxidative stress induced Twist1 expression, which resulted in the development of the CRPC phenotype [17]. In Twist1 pathways, AR expression was induced, while in the PKA pathway, AR expression was stable but AR was activated via phosphorylation [14,23]. Therefore, there might be multiple pathways including Twist1 as well as PKA pathways in the connection between oxidative stress and castration resistant phenotypes. In other words, Twist1 and PKA pathways may synergistically promote the progression of prostate cancer. In view of these observations, the reduction of ROS production might prevent the progression to CRPC.

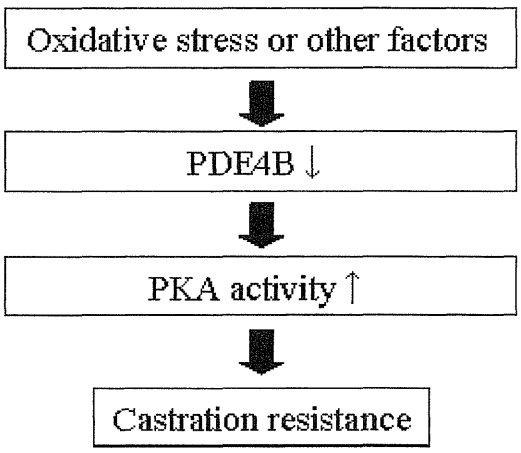

Fig. 8. Schematic representation of the relationship between PDE4B and prostate cancer phenotype. Oxidative stress or other factors downregulate PDE4B expression, activate the PKA pathway and make the prostate cancer to castration-resistant phenotype.

CONCLUSION

The current study illustrated that PDE4B expression was reduced and the activity of PKA was enhanced in CRPC. Moreover, we revealed that decreased PDE4B resulted in activation of the PKA pathway, leading to AR transactivation in prostate cancer. Oxidative stress can induce PDE4B downregulation and activate PKA signals, which could have critical functions in obtaining castration resistance and prostate cancer progression. Taken together, PDE4B/PKA pathway might represent an attractive therapeutic molecular target in the treatment of prostate cancer.

ACKNOWLEDGMENTS

We would like to thank Dr Dongchon Kang (Kyushu University, Fukuoka, Japan) for helping with quantitative real-time PCR, and Noriko Hakoda and Seiko Kamori for their technical assistances.

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Review

Cell PRESS

Two opposing roles of *O*-glycans in tumor metastasis

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Despite the high prevalence of metastatic cancers and the poor outcome for patients, the processes of tumor metastasis still remain poorly understood. It has been shown that cell-surface carbohydrates attached to proteins through the amino acids serine or threonine (*O*-glycans) are involved in tumor metastasis, with the roles of *O*-glycans varying depending on their structure. Core2 *O*-glycans allow tumor cells to evade natural killer (NK) cells of the immune system and survive longer in the circulatory system, thereby promoting tumor metastasis. Core3 *O*-glycans or *O*-mannosyl glycans suppress tumor formation and metastasis by modulating integrin-mediated signaling. Here, we highlight recent advances in our understanding of the detailed molecular mechanisms by which *O*-glycans promote or suppress tumor metastasis.

Two opposing roles of carbohydrates in tumor metastasis

Extensive investigation of cell-surface carbohydrates in tumor cells has revealed two opposing roles in tumor metastasis: depending on their structure, carbohydrates can either promote or suppress metastasis. Cell-surface carbohydrates attached to proteins are classified by the nature of their linkages to the protein as either *N*-glycans (*N*-acetylglucosamine attached to asparagine) or *O*-glycans (*N*-acetylgalactosamine attached to serine or threonine).

It has been shown that *N*-glycans play several important roles in tumor metastasis [1]. For example, upregulation of the *N*-glycan branching enzyme β-1,6-*N*-acetylglucosaminyltransferase V reduced cell–cell interactions within a tumor, promoting cell detachment and invasion by tumor cells [2]. β-1,6-*N*-acetylglucosaminyltransferase III suppressed lung metastasis of melanoma cells by inhibiting tumor cell attachment to extracellular matrices in the extravasation stage of metastasis [3]. It was recently reported that HNK-1 glycan suppresses astrocytic tumor invasion and metastasis [4].

Although it has been suggested that *O*-glycans are also involved in the process of tumor metastasis [5], the detailed molecular mechanisms have been largely unknown. In fact, the roles of *O*-glycans in tumor metastasis vary, depending on their structure. In this review, we summarize the roles of three different *O*-glycans in tumor metastasis.

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Structures of *O*-glycans involved in tumor metastasis

In mammalian cells, biosynthesis of *O*-glycans starts with the transfer of *N*-acetylgalactosamine (GalNAc) or mannose (Man) to a polypeptide (Figure 1). GalNAc is transferred to a serine or threonine residue in the polypeptide by peptide GalNAc transferase (GalNAc-T). The first GalNAc can then be extended with additional sugars, including galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose, or sialic acid (Neu5Ac). There are four common *O*-glycan core structures in mammalian tissues, core1–core4, that depend on the combination of sugars added (Figure 1).

To generate the core1 or core2 structures the initial GalNAcα1-Ser/Thr is first converted by core1 synthase to Galβ1-3GalNAcα1-Ser/Thr (core1). Core1 is then converted to core2 by β-1,6-*N*-acetylglucosaminyltransferase (core2 β1-6 GlcNAc transferase, or C2GnT). There are three enzymes that can synthesize core2, C2GnT-1, -2 and -3 (Figure 1). Core2 *O*-glycan is a scaffold for the subsequent production of lactosamine repeats (poly-*N*-acetyllactosamine) on *O*-glycans. Poly-*N*-acetyllactosamine formation is a critical step for the terminal modification by sialic acid and fucose [6–8]. The carbohydrates that contain sialic acid or fucose perform important roles in the various steps of tumor formation and tumor progression [9,10]. Enhanced expression of certain sialic acid types or their linkages often correlates with poor prognosis for many human malignancies [11,12]. For instance, fibrosarcoma cells use α2,6-linked sialic acid to evade tumor immune surveillance systems [13].

Core3 *O*-glycan is synthesized onto the initial GalNAcα1-Ser/Thr by core3 synthase (β3GnT-6), which adds β-1,3-linked *N*-acetylglucosamine to *N*-acetylgalactosamine at the reducing terminus [14]. Core4 *O*-glycan is then synthesized by the addition of β-1,6-*N*-acetylglucosamine to the core3 acceptor by C2GnT-2 (Figure 1) [15,16].

The importance of *O*-mannosylation is illustrated by the roles *O*-mannosyl glycans play in a variety of processes, including viral entry into cells, metastasis, cell adhesion and neuronal development [17], despite the fact that *O*-mannosyl glycans are rare in mammals and found in

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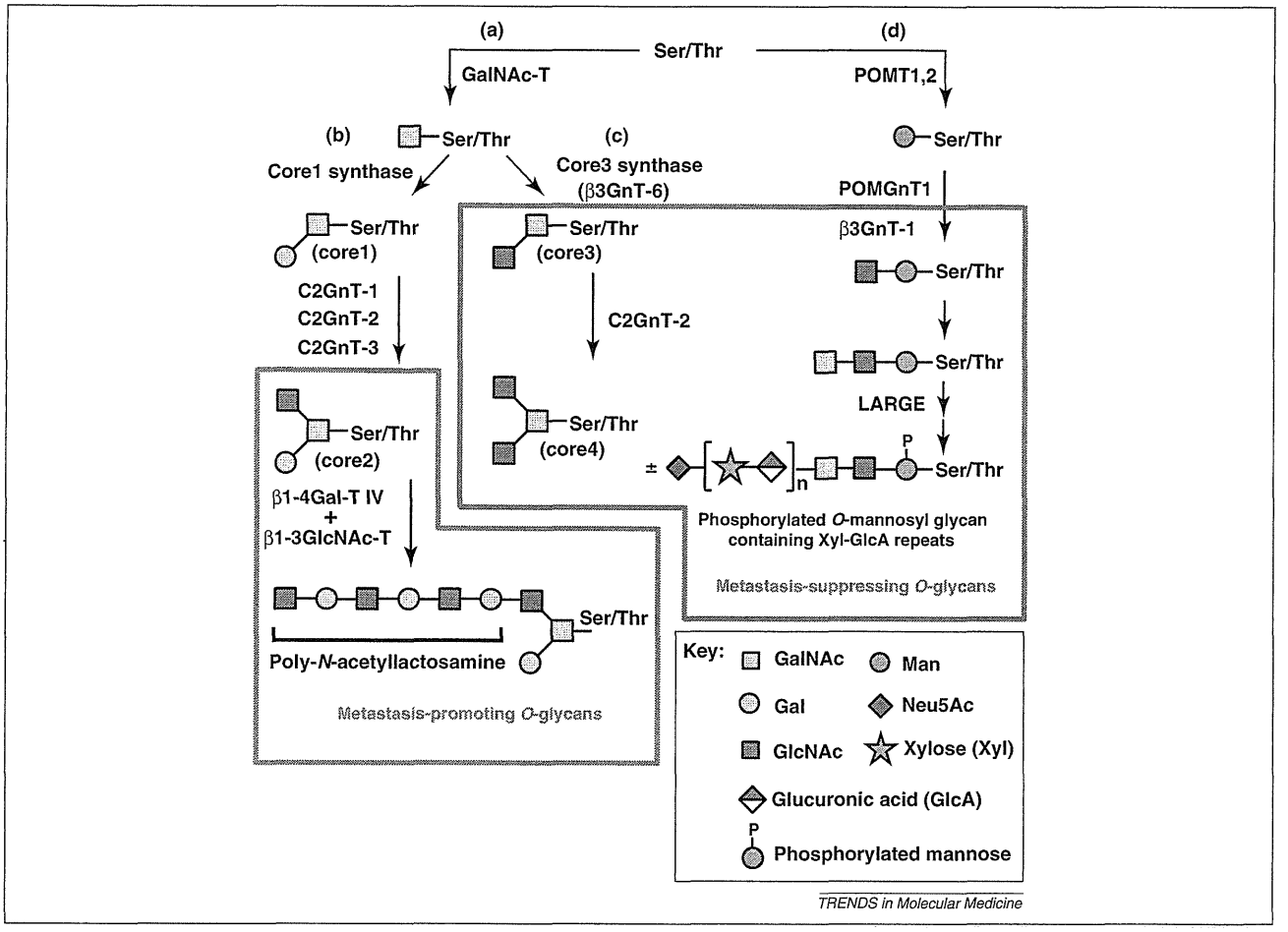

Figure 1. *O*-Glycan biosynthesis pathways that promote or suppress tumor metastasis. **(a)** To begin synthesis of core2 and core4 *O*-glycans, *N*-acetylgalactosamine (GalNAc) is transferred to a serine (Ser) or threonine (Thr) residue in a polypeptide by the peptide GalNAc transferase (GalNAc-T), as shown on the left side of the figure. GalNAcα1-Ser/Thr is then converted by core1 synthase to Galβ1-3GalNAcα1-Ser/Thr (core1) **(b)**. Core1 is converted to core2 by core2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT-1, -2 and -3). β-1,4-galactosidase IV (β1-4Gal-T IV), together with β-1,3-*N*-acetylglucosaminyltransferase (β1-3GlcNAc-T V), synthesizes poly-*N*-acetyllactosamine on core2 *O*-glycans. The number of Galβ1-4GlcNAc disaccharide unit repeats varies depending on the carrier molecules and cell types. **(c)** GalNAcα1-Ser/Thr is also converted by core3 synthase (β3GnT-6) to core3. Core3 is then converted by C2GnT-2 to core4. By contrast, synthesis of *O*-mannosyl glycans begins with transfer of mannose to a serine or threonine residue by protein *O*-mannosyltransferase 1 and 2 (POMT1 and 2) **(d)**. LARGE has α-xylosyltransferase and β-glucuronic acid transferase activities. Manα1-Ser/Thr is converted by a series of glycosyltransferases, including protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), β3GnT-1, and LARGE, to *O*-mannosyl glycan, which contains sialic acid (Neu5Ac), α-xylose, and β-glucuronic acid disaccharide unit repeats and phosphorylated mannose. As discussed in the main text, poly-*N*-acetyllactosamine-modified core2 *O*-glycans can be described as 'metastasis-promoting *O*-glycans' (bounded by the red line), and core3- and core4-containing *O*-glycans and phosphorylated *O*-mannosyl glycans can be described as 'metastasis-suppressing *O*-glycans' (bounded by the blue line).

a limited number of glycoproteins [18,19]. α-Dystroglycan (α-DG) is a highly glycosylated cell-surface molecule that functions as a linker between extracellular matrix (ECM) proteins and epithelial cells by binding to both laminin and β-DG. Binding of α-DG to laminin is mediated through a unique *O*-mannosyl glycan referred as laminin-binding glycan [20,21]. Biosynthesis of laminin-binding glycan is initiated by the transfer of mannose to a Ser or Thr residue that is catalyzed by protein *O*-mannosyltransferase 1 and 2 (POMT1 and 2) [22]. Protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) [23] and LARGE [24] then catalyze the transfer of GlcNAc to Manα1-Ser/Thr. GlcNAcβ-1,2-Manα1-Ser/Thr is finally converted to an *O*-mannosyl glycan containing Neu5Ac and phosphorylated mannose, which is apparently required for α-DG binding to laminin (Figure 1) [20]. Recent studies showed that LARGE has α-xylosyltransferase and β-glucuronic acid transferase activities, suggesting that laminin-binding glycan is composed of polymer of α-xylose and β-glucuronic acid disaccharide units [25].

The expression of core2 *O*-glycans has been detected in malignant tumors [26–31], whereas core3 and core4 *O*-glycans are synthesized in normal cells but are down-regulated in cancer cells [32,33]. In addition, it was demonstrated that *O*-mannosyl glycans are dramatically decreased in aggressive prostate and breast carcinomas [21]. These observations led to the hypothesis that core2 *O*-glycans are metastasis-promoting but that core3 and core4 *O*-glycans and *O*-mannosyl glycans are metastasis-suppressing. We explain below in more detail the recently identified mechanisms by which these *O*-glycans promote or suppress tumor metastasis.

Metastasis-promoting core2 *O*-glycans

The roles of cell-surface carbohydrates in promoting tumor metastasis have been investigated so far by focusing on

Box 1. Selectins

Selectins, a family of cell adhesion molecules, are expressed by leukocytes, endothelial cells, and platelets. All selectins are single-chain transmembrane glycoproteins that interact with cell-surface glycoconjugates and mediate tethering, rolling, and adhesion of several types of cells. There are three subsets of selectins: L-selectin is constitutively expressed by leukocytes, E-selectin by activated endothelial cells, and P-selectin by platelets and activated endothelial cells. Sialyl Lewis X [sLe(x)] and sialyl Lewis A [sLe(a)] tetrasaccharides are carbohydrate motifs displayed on protein or lipid scaffolds that are critical components of functional selectin ligand glycoconjugates.

selectin-mediated interactions between tumor cells and other cells (Box 1). Sialyl Lewis x [sLe(x)] expressed on tumor cells interacts with E-selectin on endothelial cells, and core2 *O*-glycan-based sLe(x) interacts with P-selectin on platelets. These interactions are the critical events for successful extravasation of tumor cells into metastasis target organs [34,35]. Fucosylation is a key step for the synthesis of selectin ligands and also plays a critical role in tumor progression [36,37].

Core2 *O*-glycan was originally found in activated T cells, and aberrant expression of core2 *O*-glycan was shown to be associated with immunodeficiency [38]. In addition, aberrant *O*-glycosylation associated with cancer alters the effector functions mediated by cytotoxic T lymphocytes and NK cells [39,40]. Core2-based *O*-glycans also play an important role in tumor metastasis by contributing to survival of circulating tumor cells [31].

It was reported that expression of C2GnT-1 (written as C2GnT hereafter), a key enzyme for core2 *O*-glycan expression, was closely correlated with highly metastatic phenotypes of several tumor types. Yousefi *et al.* compared C2GnT activity between highly and poorly metastatic tumor cell lines [26], and in all the tested experimental models of malignancy, C2GnT activity was elevated in highly metastatic cell lines compared with poorly metastatic cell lines, whereas there was no significant difference in activity for several other glycosyltransferases [26]. The correlation between C2GnT expression and the malignant phenotypes implies that C2GnT expression might promote tumor metastasis.

Molecular cloning of C2GnT provided useful research tools such as a DNA probe and an anti-C2GnT antibody [41,42]. To validate the above observations from tumor cell lines, several research groups examined specimens from cancer patients by immunohistochemical analysis using anti-C2GnT and *in situ* hybridization using C2GnT cDNA as a probe. The analyses revealed that C2GnT expression was detected in high-grade or highly metastatic tumors, but not in low-grade tumors or normal tissue from patients. Furthermore, C2GnT expression was closely correlated with vessel invasion in colorectal cancer [27]. Statistical analysis of C2GnT expression in pulmonary adenocarcinoma and clinicopathological variables revealed that C2GnT expression was significantly associated with vessel invasion and lymph node metastasis [28]. These results indicate that C2GnT expression is correlated with the progression of colorectal cancer and lung cancer. It was later found that C2GnT expression also positively correlates with the progression of prostate cancer, testicular germ-cell tumors, and bladder cancer [29–31].

Statistical analysis of the relationship between C2GnT expression, measured by immunohistochemical staining, and prognosis showed that prostate cancer, testicular germ-cell tumor, and bladder cancer patients with C2GnT-positive tumor cells lived for a significantly shorter time than patients with C2GnT-negative tumor cells, indicating that C2GnT expression is an excellent prognostic indicator [29–31]. The above observations strongly suggest that C2GnT expression promotes tumor metastasis. However, the detailed molecular mechanisms underlying highly metastatic phenotypes of C2GnT-expressing tumors were unknown.

Evasion of NK cell immunity by C2GnT-expressing tumor cells

The first attempt to understand the mechanism by which C2GnT expression promotes metastasis was made for bladder cancer. Tumor formation assays using immunodeficient mice (nude mice, SCID/beige mice, and NK cell-depleted nude mice) revealed that C2GnT-expressing cells were more resistant to NK cell attack than cells not expressing C2GnT. These results suggest that C2GnT-expressing bladder tumor cells possess a high ability to evade NK cell attack, resulting in longer survival after tumor cells disseminate into the host blood circulation [31]. The NK cell receptor–tumor ligand interaction mediates tumor cell rejection by host NK cells in circulation. Among NK receptors, the NK-activating receptor natural killer group 2 member D (NKG2D) plays a critical role in eliminating tumor cells. NKG2D interacts with the tumor-cell-expressed ligand MHC class I-related chain A (MICA), and NK cells are activated by the interaction. Activated NK cells secrete several apoptosis-inducing substances such as granzyme B and perforin, thereby killing target tumor cells (Figure 2a) [43]. The mechanism by which C2GnT-expressing tumor cells evade NK cell attack was investigated by focusing on the NKG2D–MICA interaction. The core2 branch is a scaffold for the subsequent production of lactosamine disaccharide repeats, poly *N*-acetyllactosamine (Galβ1-4GlcNAc)n, on *O*-glycans (Figure 1) [6]. Galectins are β-galactoside-binding proteins that have been implicated in numerous biological processes including immunity and tumor progression [44–46]. Galectin-1, -3, and -9 suppress tumor rejection responses by regulating T cell functions [47–52]. In addition, it was previously suggested that galectin-3 is involved in the survival of disseminating cancer cells in host circulation during metastasis [53]. Considering poly *N*-acetyllactosamine is present on core2 *O*-glycans on MICA and galectin-3 binds MICA through this poly *N*-acetyllactosamine, galectin-3 is likely to contribute to the survival of C2GnT-expressing bladder tumor cells in circulation. Modification of MICA *O*-glycans with poly *N*-acetyllactosamine and galectin-3 occurs on the NKG2D binding site in MICA, thereby reducing the affinity of MICA for NKG2D, severely impairing NK cell activation, and preventing the NK-mediated killing of C2GnT-expressing bladder tumor cells (Figure 2b) [31]. This masking of tumor cells promotes immune evasion of C2GnT-expressing bladder tumor cells. When tumor cells

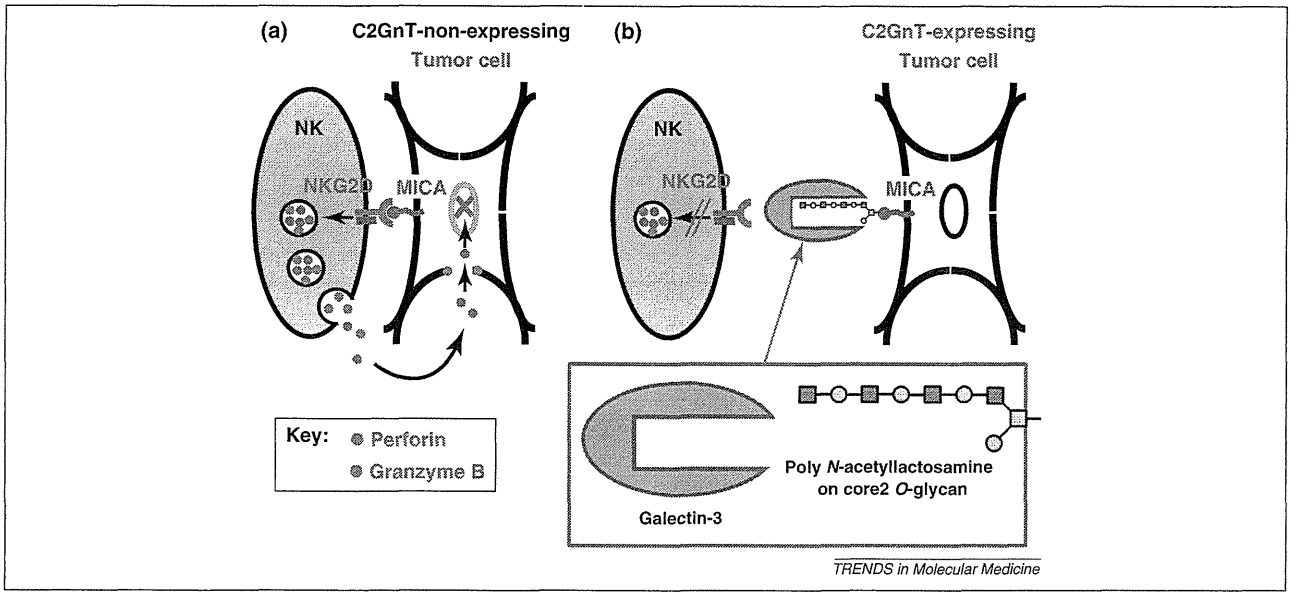

Figure 2. Core2 *O*-glycans promote tumor metastasis by evading NK cell attack. The molecular mechanism by which C2GnT-expressing tumor cells evade NK cells. **(a)** On the left is an illustration of a major mechanism by which tumors are rejected by NK cells. NK cells are activated by the interaction of NKG2D with MICA on the surface of the tumor cells. Activated NK cells secrete perforin and granzyme B to induce apoptosis of target tumor cells. **(b)** On the right is the mechanism by which C2GnT-expressing tumor cells evade NK cell attack. The protein galectin-3 binds to poly-*N*-acetyllactosamine in the NKG2D-binding site of MICA. Galectin-3 reduces the affinity of MICA for NKG2D, severely impairing NK cell activation. This masking of the tumor cells increases survival of the tumor cells in host blood circulation, promoting tumor metastasis.

were intravenously injected into nude mice, C2GnT-expressing tumor cells produced several metastatic foci in the lungs, although tumor cells not expressing C2GnT produced few foci. After poly *N*-acetyllactosamine was cleaved by endo-β-galactosidase and galectin-3 was removed from MICA in C2GnT-expressing tumor cells, the tumor cells became susceptible to NK cell attack and produced few metastatic foci [31]. Thus, both *in vitro* and *in vivo* evidence confirms that modification of MICA with poly *N*-acetyllactosamine and galectin-3 allows C2GnT-expressing tumor cells to evade NK cell attack, resulting in longer survival of the tumor cells in host circulation and promoting tumor metastasis. Considering that this novel evasion system was demonstrated in bladder tumors [31] and that C2GnT expression correlates with a highly metastatic phenotype in several other types of tumor [26–30], it is highly likely that such tumors also use this evasion system.

Analyses of NKG2D knockout mice revealed that the NKG2D–MICA interaction is critical in tumor rejection responses [54,55]. Previous studies reported that some tumor cells modulate NKG2D-mediated tumor immunosurveillance in the following three ways: a large amount of soluble MICA shed by tumor cells downregulates NKG2D expression [56–58]; tumor cells sustain localized expression of the NKG2D ligands [59]; and tumor cells decrease the cell-surface expression of MICA by retaining MICA within cells [60]. Modification of MICA with poly *N*-acetyllactosamine and galectin-3 is the fourth and newly identified mechanism by which tumor cells modulate the NKG2D–MICA interaction to evade NK cell immunity using cell-surface carbohydrates [31].

It has been reported that core2 *O*-glycans are abundant in normal breast tissues and are decreased in tumor formation [5,61], suggesting that core2 *O*-glycans do not necessarily play important roles in breast tumor formation. However, the roles of breast tumor cell-surface *O*-glycans in metastasis remain poorly understood. Future studies on the relationship between the decrease in core2 *O*-glycans and breast tumor metastasis will provide new insights into the role of cell-surface carbohydrates in tumor metastasis.

Metastasis-suppressing core3 and core4 *O*-glycans

In contrast with C2GnT, core3 synthase expression is generally observed in normal tissues, but its expression is downregulated in cancer cells, including gastric and colorectal carcinomas [14,32,33]. The expression of C2GnT-2, which is responsible for the formation of core4 *O*-glycan, is also downregulated in cancer cells [62]. Based on these observations, it was speculated that core3 and core4 *O*-glycans suppress tumor metastasis.

To determine the roles of core3 *O*-glycans in tumor metastasis, two metastatic prostate cancer cell lines, PC3 and LNCaP, were transfected with core3 synthase cDNA and examined for various phenotypes. Forced expression of core3 synthase in PC3 and LNCaP cells reduced cell migration and invasion *in vitro* [63]. Core3-synthase-expressing PC3 cells produced much smaller tumors in mouse prostates without metastasizing to surrounding lymph nodes, as compared with mock-transfected PC3 cells, when tumor cells were inoculated into immunodeficient mouse prostates. Moreover, core3-synthase-expressing LNCaP cells barely produced subcutaneous tumors, in contrast to mock-transfected LNCaP cells, when tumor cells were subcutaneously inoculated into immunodeficient mice [63]. These results from both *in vitro* and *in vivo* systems suggest that core3 synthase expression suppresses tumor

Box 2. Integrin and integrin-mediated signaling

Integrins are cell-surface receptors that recognize and bind to extracellular matrix proteins and counter-receptors. Integrins are heterodimers composed of α and β subunits. There are 18 α subunits and 8 β subunits that assemble in 24 different combinations with overlapping substrate specificity and cell-type-specific expression patterns. Binding of activated integrins to their ligands mediates a large number of structural and signaling changes within the cell. Immediate intracellular changes are increased tyrosine phosphorylation of specific substrates such as Src, focal adhesion kinase (FAK), and p130Cas, as well as an increase in the concentrations of lipid second messengers such as phosphatidylinositol-4, 5-bisphosphate. The immediate effects initiate activation of signaling pathways via serine/threonine protein kinases such as ERK, AKT, and JNK and GTPases such as Rho. These signaling pathways ultimately lead to cell survival, proliferation, differentiation, and migration.

formation and tumor metastasis. However, the mechanisms by which core3 and core4 *O*-glycans decrease cell invasion and suppress tumor metastasis were not understood.

Integrins, a class of important cell-surface receptors (Box 2), carry various types of glycans. α2β1 integrin carrying *O*-glycans is a collagen receptor and one of the major players in promoting tumor metastasis by mediating tumor cell adhesion, migration and invasion (Figure 3a) [64]. Thus, α2β1 integrin was the focus for determining the role of core3 *O*-glycans in tumor metastasis. Analyses of the prostate cancer cells discussed above revealed that the cell-surface expression of α2β1 integrin was significantly decreased in core3-synthase-expressing tumor cells [63], and it was shown that the integrin β1 subunit carries mucin-type *O*-glycans [65]. Taken together, these results indicate that core3 *O*-glycans on the β1 subunit reduce the cell-surface expression levels of α2β1. There are two possible causes of reduced levels of α2β1 (Figure 3). One is that attachment of core3 *O*-glycans hinders proper *N*-glycosylation of the β1 subunit, resulting in expression of the immature glycoform and reduced cell-surface levels of α2β1 [63]. It has been observed that protein glycosylation state influences cell-surface expression levels of integrins and their downstream signal transduction pathways. For instance, loss of *N*-glycan on the β-propeller domain of the integrin α5 subunit decreases the cell-surface expression of α5β1 [66,67], and lack of *N*-glycosylation on β4 integrin suppresses ERK- and EGFR-mediated signaling [68]. The second cause could be that core3 *O*-glycans inhibit association of the β1 subunit with the α2 subunit, resulting in reduced cell-surface levels of the mature α2β1 complex [63].

One of the α2β1 integrin-mediated signaling pathways that regulate cell migration involves focal adhesion kinase (FAK) [64]. Reduced cell-surface expression of α2β1 subsequently decreases FAK phosphorylation, impairing FAK-mediated signal transduction [63]. Impaired signaling led to reduced lamellipodia formation and cell migration. Reduced cell migration led to decreased cell invasion, suppressing tumor formation and metastasis (Figure 3b). This is the currently accepted model for how core3 synthase expression suppresses tumor metastasis.

It is likely that core3 synthase expression plays important roles in maintaining tissue homeostasis by blocking tumorigenesis in normal tissues and that its downregulation in cancer cells promotes both tumor formation and metastasis by increasing cell migration and invasion.

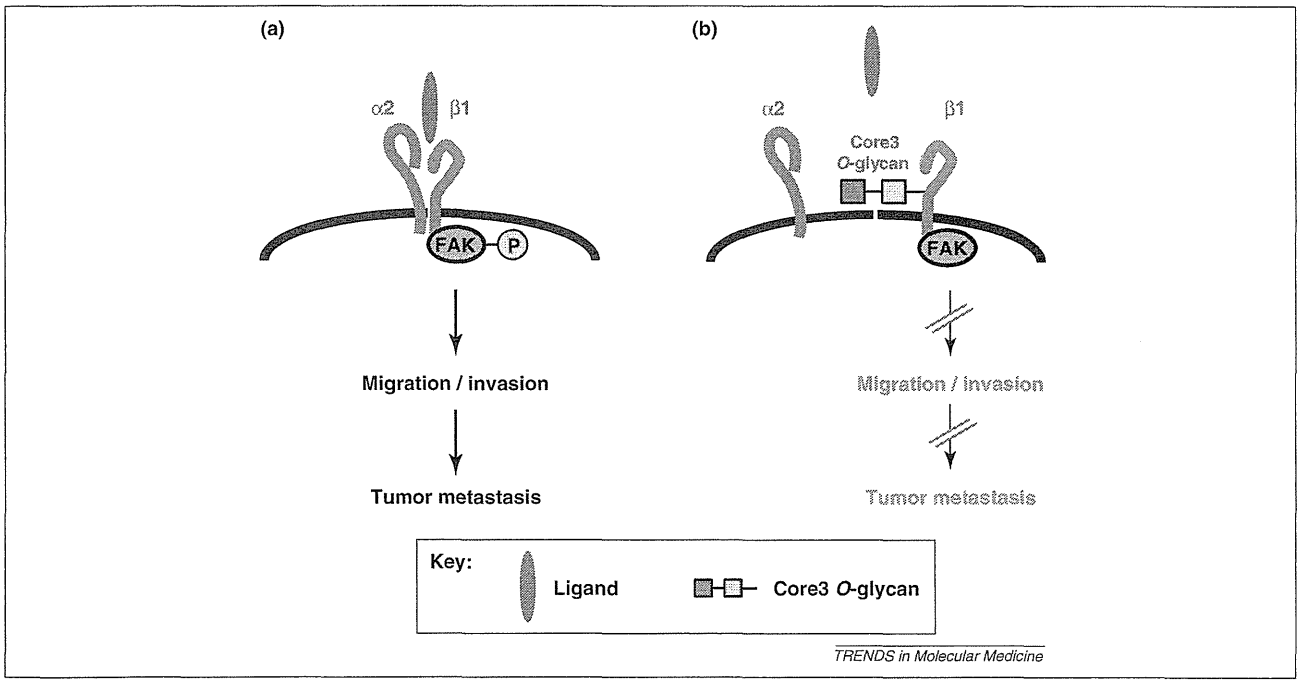

Figure 3. The role of core3 *O*-glycan in suppressing migration and tumor metastasis. **(a)** In tumor cells with low core3 synthase expression, ligand binding to α2β1 integrin induces phosphorylation of FAK. Phosphorylated FAK transduces signals to mediate tumor cell migration and invasion, promoting metastasis. **(b)** In cells that express core3 synthase, core3 *O*-glycans on the β1 subunit of α2β1 integrin prevent the association of the α2 and β1 subunits, reducing the amount of functional α2β1 complex on the cell surface. The reduced levels of α2β1 limit FAK phosphorylation, impairing signal transduction. Impaired signaling reduces cell migration and invasion and suppresses tumor formation and metastasis.

Forced expression of core3 synthase and C2GnT-2 in tumor cells significantly decreases cell invasion and tumor formation *in vivo* [14,62]. In core3-synthase-deficient mice, colon carcinoma formation after treatment with the colon carcinoma-inducing chemicals azoxymethane and dextran sodium sulfate was greatly promoted compared with wild-type mice [69]. These studies using *in vivo* models strongly support the tumor-suppressing functions of core3 *O*-glycans.

Metastasis-suppressing *O*-mannosyl glycans

The discovery of another metastasis-suppressing *O*-glycan unexpectedly came from studies of muscular dystrophy. α-DG is a highly glycosylated cell-surface molecule that is expressed in the epithelial cell-basement membrane (BM) interface and functions as a linker between ECM proteins and epithelial cells by binding to both laminin and β-dystroglycans (Figure 4). Binding of α-DG to laminin is mediated through a unique *O*-glycan carried by α-DG, the laminin-binding glycan, which has been shown to contain the core portion of sialic acid, α-xylose-β-glucuronic acid disaccharide unit repeats and phosphorylated mannose (Figure 1) [18,25]. Laminin interaction with α-DG through the laminin-binding glycan is critical for assembling BM. Therefore, defects in glycosylation of this glycan, which requires several glycosyltransferase-like genes, including *POMT1*, *POMT2*, *POMGnT1* and *LARGE* [22], cause muscular dystrophy [23,24,70].

LARGE is a causative gene product for muscular dystrophy in both mice and humans [71–73] and contains a domain homologous to β3-*N*-acetylglucosaminyltransferease 1 (β3GnT1) [71]. β3GnT1 synthesizes i-antigen, a linear poly *N*-acetyllactosamine on human fetal red blood cells [74]; however, the physiological role of β3GnT1 was unknown. On the basis of the structural similarity of β3GnT1 to LARGE, a role for β3GnT1 in the biosynthesis of laminin-binding glycans was investigated.

An important role for laminin-binding glycans in tumor metastasis was first demonstrated in prostate and breast cancers [21]. In aggressive human prostate and breast cancer cells, laminin-binding glycans of α-DG are dramatically decreased, although the amount of α-DG core protein is not changed [21]. The decrease in laminin-binding glycans was associated with decreased expression of β3GnT1. Forced expression of β3GnT1 in the aggressive cancer cells restored synthesis of laminin-binding glycans and reduced cell migration and invasion *in vitro* [21]. Moreover, β3GnT1-expressing PC3 cells inoculated into the prostate of immunodeficient (SCID) mice produce much smaller tumors than mock-transfected PC3 cells [21]. These results suggest that laminin-binding glycans synthesized by β3GnT1 are involved in suppressing tumor formation and metastasis.

The β1 integrins such as α2β1 and α9β1 mediate ECM-induced cell motility by activating several signaling pathways including the ERK–AKT pathway. Downregulation of β3GnT1 in prostate cancer cells increases phosphorylation of AKT and ERK, promoting ECM-mediated cell migration [21]. Conversely, phosphorylation of ERK and AKT was much lower in β3GnT1-expressing tumor cells than mock-transfected cells [21], suggesting a molecular

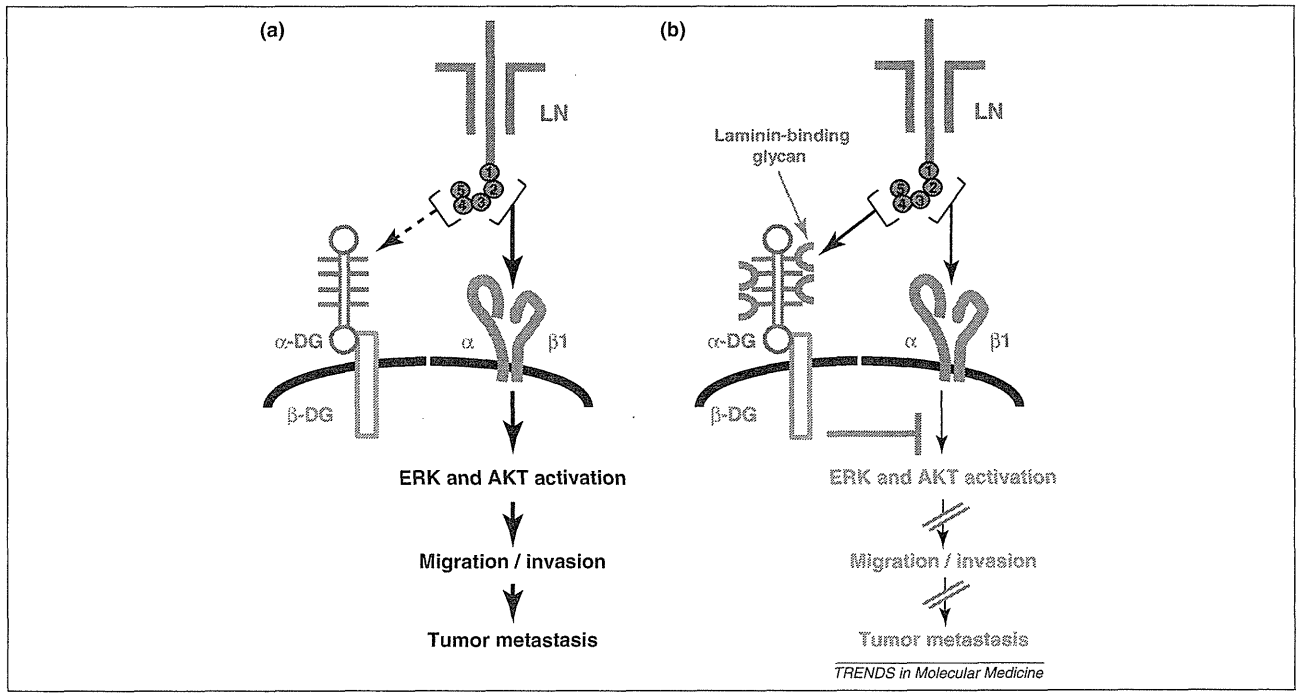

Figure 4. The role of phosphorylated *O*-mannosyl glycan (laminin-binding glycan) in suppressing tumor metastasis. **(a)** In tumor cells with downregulated β3GnT1 expression, laminin (LN) globular domains 4 and 5 are unable to bind to α-DG because the α-DG laminin-binding glycan is not synthesized (denoted by broken arrow), but laminin globular domains 1, 2 and 3 still bind to β1 integrin (denoted by bold and solid arrow), activating ERK–AKT signaling. This activated signaling mediates high tumor migration and invasion, resulting in metastasis. **(b)** In β3GnT1-expressing tumor cells, laminin-binding glycan containing α-xylose and β-glucuronic acid disaccharide unit repeats and phosphorylated mannose is synthesized on α-DG. Laminin globular domains 4 and 5 bind to α-DG through laminin-binding glycan (denoted by solid arrow), decreasing the amount of laminin available to bind β1 integrin (denoted by thin and solid arrows) and counteracting ERK–AKT signaling. This results in suppressed tumor migration and metastasis. Globular domains 1–5 of the laminin α-chain are indicated by the circled numbers.

mechanism by which β3GnT1 expression suppresses tumor migration and metastasis (Figure 4a). In aggressive tumor cells with downregulated β3GnT1 expression, the laminin globular domains 4 and 5 of laminin α-chain are unable to bind to α-DG because of a lack of laminin-binding glycan (denoted by the broken arrow in Figure 4a), but laminin globular domains 1, 2, and 3, as well as other ECM proteins, can still bind to the β1 integrin (denoted by bold solid arrows in Figure 4a), activating the ERK–AKT signaling pathway and leading to high levels of tumor migration and metastasis [75,76]. By contrast, the laminin-binding glycan is synthesized in β3GnT1-expressing tumor cells and a portion of the laminin population binds to α-DG as well as the integrins (denoted by thin solid arrows in Figure 4b), resulting in decreased laminin binding to the β1 integrin. Laminin binding to α-DG therefore counteracts the ERK–AKT signaling pathway, leading to low tumor migration and metastasis (Figure 4b) [21]. Future studies will examine the mechanism of β3GnT1 downregulation in normal tissues and tumor cells with low metastatic potential. The above results also indicate that glycosyltransferases that synthesize laminin-binding glycans are coordinately regulated, as seen for LARGE and β3GnT. Using siRNA library screening, recent studies revealed that Fer kinase downregulates the expression of LARGE and β3GnT1 [77]. Because Fer kinase also functions as an oncogene, this finding supports the conclusion that laminin-binding glycans function as tumor suppressors.

Other *O*-glycosylated proteins involved in tumor metastasis

There are several other observations suggesting that *O*-glycosylation plays important roles in tumor metastasis. Two studies of human colon cancer cells implicate sialylated *O*-glycans in tumor metastasis. Removal of sialic acids from the β4 integrin *O*-glycans by the sialidase NEU1 decreased phosphorylation of the integrin, resulting in suppression of migration and metastasis [78], and removal of sialic acids from mucin *O*-glycans by another sialidase, NEU4, also suppressed adhesion and migration of colon cancer cells [79]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) stimulates cancer cell death through the proapoptotic receptors DR4 and DR5. *O*-Glycosylation of the death receptors is critical in controlling apoptotic signaling [80]. Finally, increased *O*-glycosylation of oncofetal fibronectin is required for the epithelial–mesenchymal transition (EMT) process, a key step in tumor progression and metastasis [81].

Concluding remarks

This short review, with an emphasis on the most recent advances, provides several examples that support the significance of *O*-glycans in tumor metastasis. *O*-Glycans play two opposing roles in tumor metastasis depending on their structures. *O*-Glycans containing a core2 branch promote tumor metastasis by impairing NK cell activation [31]. By contrast, *O*-glycans containing core3 or phosphorylated *O*-mannosyl glycans suppress tumor metastasis by modulating integrin-mediated signaling [21,63].

Our current understanding of the molecular mechanisms by which those *O*-glycans promote or suppress tumor metastasis may lead to the development of new therapeutic methods and agents for preventing tumor metastasis. Reduction of C2GnT activity or downregulation of C2GnT expression using specific inhibitors or siRNAs in tumor cells could restore tumor cell susceptibility to NK cell attack, promoting tumor rejection and suppressing tumor metastasis. Increased expression of core3 synthase and β3GnT1 in tumor cells would reduce integrin-mediated signaling, suppressing both tumor migration and metastasis.

Further studies on the structures and functions of *O*-glycans, focused on the process of tumor metastasis, will provide a new insight into the roles of cell-surface carbohydrates in tumor metastasis.

Acknowledgments

We thank the Japan Society for the Promotion of Science for their support (grant 22570131 to S.T. and B22390301 to C.O.), as well as the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant 21791483 to C.O.), the Japan Science and Technology Agency (CREST to C.O.) and the National Institutes of Health (grants P01CA71932 and R01CA3000 to M.F.).

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Prostate Cancer and Prostatic Diseases (2012) 15, 397–401

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ORIGINAL ARTICLE

Safety and effectiveness of neoadjuvant luteinizing hormone-releasing hormone agonist plus low-dose estramustine phosphate in high-risk prostate cancer: a prospective single-arm study

T Koie, C Ohyama, H Yamamoto, S Hatakeyama, T Yoneyama, Y Hashimoto and N Kamimura

BACKGROUND: Radical prostatectomy (RP) has limited cancer control potential for the patient with high-risk prostate cancer (Pca). We prospectively examined the efficacy and safety of neoadjuvant therapy with luteinizing hormone-releasing hormone (LHRH) agonist + low-dose estramustine phosphate (EMP) (LHRH + EMP) followed by RP.
METHODS: High-risk Pca was defined by the D'Amico stratification system. A total of 142 patients with high-risk Pca were enrolled in this trial from September 2005 to March 2011. The LHRH + EMP therapy included administration of LHRH agonist and 280 mg day^{-1} EMP for 6 months before RP. Pathological cancer-free (pT0) rate on the surgical specimen was the primary end point. Secondary end points were PSA-free survival and toxicity.
RESULTS: The average patient age was 67.4 years (interquartile range (IQR) 72, 65) and the median initial PSA level was 14.80 ng ml^{-1} (IQR 26.22, 7.13). The median Gleason score was 9 (IQR 9, 7) and 97 patients (68.3%) had clinical stage T2c or T3. All patients completed 6 months of LHRH + EMP neoadjuvant therapy with no delays in RP. Seven patients (4.9%) achieved pT0. Surgical margins were negative in 125 patients (87.0%). At a median follow-up period of 34.9 months, PSA-free survival was 84.3%. No serious adverse events were reported during the study and there were no toxicity-related deaths.
CONCLUSIONS: Six months of LHRH + EMP neoadjuvant therapy followed by RP is safe and oncological outcomes are acceptable. Although this study was a single-arm trial with a relatively short follow-up, this treatment may have a potential to improve PSA-free survival in high-risk Pca patients. Further clinical trials are warranted.

Prostate Cancer and Prostatic Diseases (2012) **15,** 397–401; doi:10.1038/pcan.2012.29; published online 14 August 2012

Keywords: high-risk; neoadjuvant therapy; luteinizing hormone-releasing hormone agonist plus low-dose estramustine phosphate

INTRODUCTION

According to the D'Amico risk stratification, patients who have PSA levels of 20 ng ml^{-1} or greater, Gleason scores of 8 or higher, or clinical stage T2c/T3 are defined as high-risk localized or locally advanced prostate cancer (Pca).[1] In this group of patients, reported rates of PSA-free survival after local therapy range from 30 to 50%.[1,2]

Neoadjuvant hormonal therapy alone before radical prostatectomy (RP) reduces the rate of positive surgical margins and can achieve a pathological complete response. However, neoadjuvant androgen deprivation therapy has shown no benefits in patient outcomes, especially PSA-free survival, in randomized trials.[3–6] Several studies have reported similar results with neoadjuvant docetaxel in high-risk Pca patients.[7–10] Although various trials try to achieve pT0 or prolonged PSA-free survival, the pT0 rate was ranged from 0 to 2.8%[9–11] and the PSA-free survival rate was still low.[8,12]

We thought that other combination with hormonal and cytotoxic agents may contribute to improve oncological outcome of high-risk Pca. Estramustine phosphate (EMP) is a compound of estrogen and nitrogen mustard, which deregulates microtubule assembly.[13,14] We have reported the active effect of luteinizing hormone-releasing hormone (LHRH) plus EMP for the patients with advanced Pca.[15,16] We expected that this combination may be applicable to neoadjuvant therapy for the patients with high-risk Pca.

In this prospective study, we assessed the activity and safety of neoadjuvant LHRH plus low-dose EMP (LHRH + EMP) before RP in patients with high-risk Pca.

MATERIALS AND METHODS

Study population

This was a prospective single-arm study carried out at a teaching hospital in Japan. All enrolled patients were Japanese. High-risk Pca was defined as clinical stage T2c or T3, and/or initial PSA levels of 20 ng ml^{-1} or greater, and/or a biopsy Gleason sum score of 8 or higher according to the D'Amico risk stratification system.[1] Eligible patients had histologically confirmed high-risk Pca without lymph node or distant metastasis, an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1, adequate bone marrow function (absolute neutrophil count $\geqslant$1500 m^{-3} and platelet count $\geqslant$100 000 m^{-3}), adequate renal function (creatinine $<$2.0 mg dl^{-1} and/or creatinine clearance $>$40 ml min^{-1}), and adequate hepatic function (total bilirubin $<$1.5 mg dl^{-1}). Patients who underwent prior radiation therapy for the prostate or pelvis, or had received chemotherapy or hormonal therapy for Pca were not enrolled. The patients who administered finasteride or dutasteride before surgery were excluded in this study.

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Received 23 February 2012; revised 22 June 2012; accepted 24 June 2012; published online 14 August 2012

Table 1. Clinical characteristics of patients administered neoadjuvant therapy with LHRH agonist plus estramustine phosphate

Initial PSA level (ng ml^{-1}) (N, %)	
<20	84 (59.2)
⩾20	58 (40.8)
Median (IQR)	14.80 (7.13–26.23)
Clinical stage (N, %)	
T1c	20 (14.1)
T2a	11 (7.7)
T2b	14 (9.9)
T2c	26 (18.3)
T3	71 (50)
Biopsy Gleason score (N, %)	
⩽6	4 (2.8)
7	43 (30.3)
⩾8	95 (66.9)
Number of positive biopsy cores (IQR)	4 (3–6)

Abbreviations: IQR, interquartile rate; LHRH, luteinizing hormone-releasing hormone.

The present neoadjuvant therapy achieved 4.6% of pT0 at our preliminary study.[17] The highest pT0 rate ever reported was 2.8%.[9] Therefore, we expected the highest pT0 rate for the neoadjuvant therapy as around 5% in this study.

All patients were informed about the treatment protocol, and provided written consent. The study protocol and informed consent documents were reviewed and approved by the Hirosaki University institutional review board.

Treatment

All treatments in this study were undertaken at our institution. All patients received LHRH agonist (leuprolide 11.25 mg or goserelin acetate 10.8 mg every 3 months) and EMP 280 mg day^{-1} for 6 months before RP. The use of our retropubic retrograde technique with RP was previously described in detail.[18] All patients in this study underwent the same lymphadenectomy procedure, which included removal of the bilateral obturator lymph node chains.

Patient evaluation

Baseline evaluation included complete history and physical examination, assessment of ECOG performance status, serum testosterone and PSA levels, abdominal and pelvic computed tomography or magnetic resonance imaging, and chest X-ray or computed tomography.

Toxicity was evaluated after neoadjuvant LHRH + EMP every 3 months and graded by the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Prostatectomy specimens were evaluated by a single pathologist at our institution according to International Society of Urological Pathology 2005.[19] The apex of the prostate was shaved perpendicular to the prostatic urethra. The bladder neck margin was coned from the specimen and sectioned perpendicularly. The remaining prostate was completely sectioned at 3 mm intervals in a plane perpendicular to the urethral axis. All tumors were staged by the 2002 American Joint Committee on Cancer staging manual.[20] Absence of evidence of cancer in the prostate and lymph node specimens was classified as a pT0.

Follow-up schedule

Following surgery, all patients were assessed by measuring serum PSA and testosterone at 3-month intervals. The date of disease recurrence or PSA failure was defined as when the serum PSA level exceeded 0.2 ng ml^{-1}. If PSA did not decrease to <0.2 ng ml^{-1} after surgery, the date of RP was defined as date of disease recurrence.

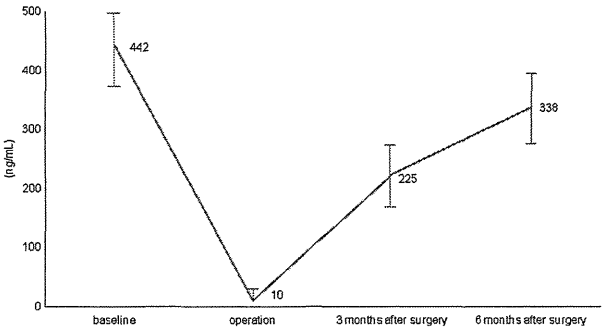

Figure 1. Change in serum testosterone levels. The median serum testosterone level was 442 ng ml^{-1} at baseline. The nadir serum testosterone level was 10 ng ml^{-1} (interquartile range 15, 5) at the time of operation. The median serum testosterone levels 3 and 6 months after surgery were 225 and 338 ng ml^{-1}, respectively.

Statistical analysis

Data were analyzed using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA). PSA-free survival was analyzed by the Kaplan–Meier method. The relationship between survival and subgroup classification was analyzed using the log-rank test. All *P*-values were two-sided, and the significance level was set at $P<0.05$. The primary end point was the percentage of patients showing pT0. The secondary end points were PSA-free survival and toxicity.

RESULTS

Patient characteristics

From September 2005 to March 2011, 142 patients with high-risk Pca were enrolled. The pre-treatment characteristics of these patients are listed in Table 1. The mean patient age was 67.4 years (interquartile range (IQR) 72, 65). All patients received neoadjuvant LHRH + EMP according to the aforementioned protocol.

Surgical outcomes

All patients completed neoadjuvant therapy and underwent RP. Deep vein thrombosis or pulmonary embolism was not seen in our series. The median operative time for RP including bilateral lymphadenectomy was 110 min (IQR 129, 94) and median estimated blood loss was 853 ml (IQR 1285, 610). The median number of removed lymph nodes was 8 (IQR 12, 4).

We experienced one rectal injury (0.7%) with intraoperative complication, which was successfully repaired. Leakage from the vesicourethral anastomosis, which is the most frequent perioperative complication, occurred in 26 patients (18.3%), and surgical site infection occurred in 3 (2.1%).

Change in serum testosterone level

Chronological change in serum testosterone levels ± s.d. was shown in Figure 1. The median serum testosterone level was 442 ± 133 ng ml^{-1} (IQR 516, 375) at baseline. Serum testosterone level had decreased to castration levels in all patients within 3 months after starting neoadjuvant therapy and maintained <50 ng ml^{-1} until RP. Serum testosterone level became <20 ng ml^{-1} in 107 patients (75.3%). The nadir serum testosterone level was 10 ± 10 ng ml^{-1} (IQR 15, 5) at the time of operation. The median serum testosterone levels 3 and 6 months after surgery were 225 ± 78 and 338 ± 125 ng ml^{-1}, respectively.

Pathological outcomes

The estimated preoperative prostate volume on transrectal ultrasonography was 29.1 ml (IQR 37.9, 22.3). The median surgical

specimen weight was 19.8 g (IQR 25.0, 14.5). All patients were evaluated for pathological response. As to pathological T stage, 4.9%, 56.3% and 38.7% of patients had pT0, pT2 and pT3, respectively.

Overall, 17 patients (13%) had positive surgical margins, including 0% of those with pT0, 1.3% with pT2 and 29.1% with pT3 disease. Lymph node involvement was detected in 2 (1.4%) patients.

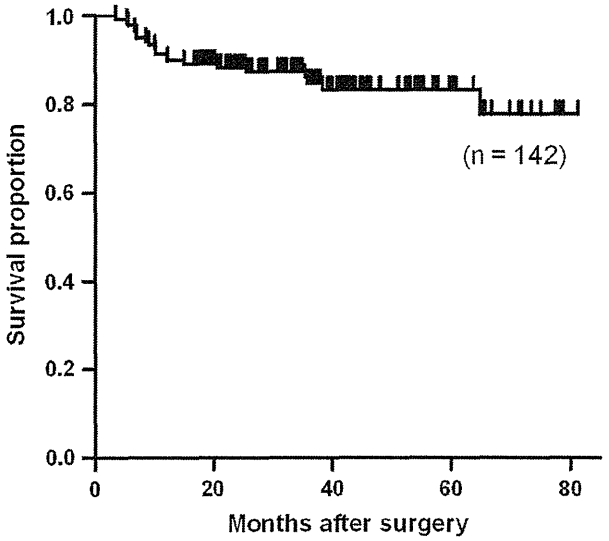

Figure 2. Kaplan–Meier estimate of PSA-free survival. At 34.5 months, the PSA-free survival rate was 86.0%.

Oncological outcomes

The postoperative mean follow-up period was 34.9 months (IQR 51.6, 21.0). During follow-up, PSA relapse without clinical recurrence occurred in 21 patients (14.8%). Clinical recurrence occurred in two patients (1.4%). At 34.9 months, the PSA-free survival rate was 86.0% (95% confidence interval (CI), 56.4–65.4) (Figure 2). The 3-year PSA-free survival rate was 96.9% for patients in clinical stage T1c to T2b (95% CI, 33.5–36.6) and 78.5% for patients in clinical stage T2c or greater (95% CI, 53.2–63.9; $P = 0.035$) (Figure 3a).

The 3-year PSA-free survival rate was 91.5% in patients who achieved pathological T0 to T2 status (95% CI, 60.1–67.4) and 68.9% in those with T3 status (95% CI, 42.8–60.0; $P = 0.001$) (Figure 3b). The 3-year PSA-free survival rate was 86.9% in patients with negative surgical margins (95% CI, 61.2–69.1) and 53.0% in those with positive surgical margins (95% CI, 23.3–45.7; $P < 0.0001$) (Figure 3c).

Safety of neoadjuvant LHRH plus EMP

No serious adverse events were reported during the study and there were no toxicity-related deaths. No grade 3 or 4 toxicities occurred (Table 2). The most common LHRH + EMP-related adverse event was grade 1 gynecomastia (98.6%). All adverse events occurred within 3 months after administration of LHRH + EMP.

DISCUSSION

According to previous literatures, pT0 rate have been reported to range from 0 to 2.8%.[9–11] To achieve better oncologic outcome of high-risk Pca is one of the major concerns in urological oncology.[21] The results with phase II studies using docetaxel-based neoadjuvant therapy have been indicated that pathological cancer-free status and acceptable PSA-free survival can rarely be achieved although gefitinib was added on

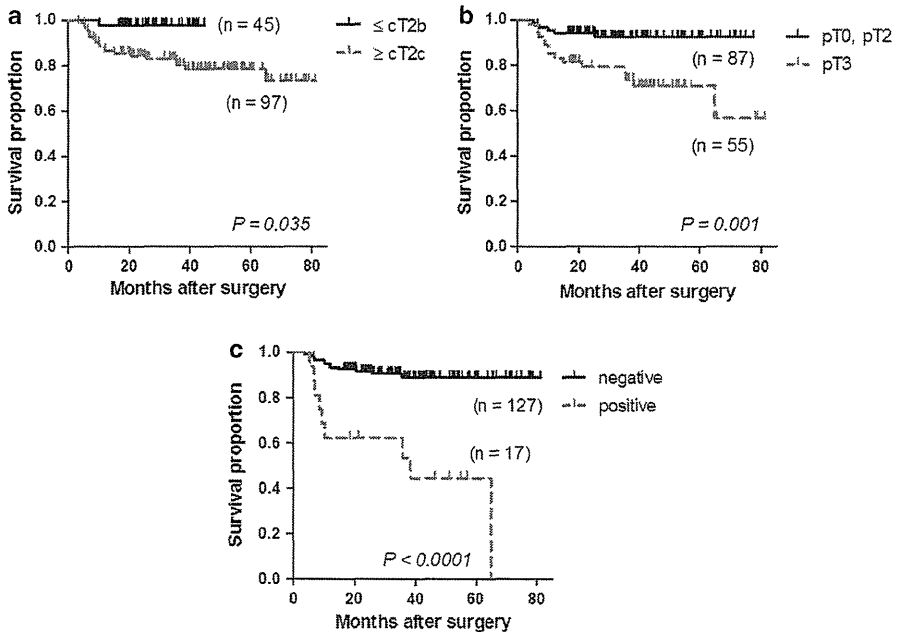

Figure 3. Kaplan–Meier estimate of PSA-free survival. (**a**) Kaplan–Meier estimate of PSA-free survival according to clinical stage. The 3-year PSA-free survival rate was 96.9% for patients in clinical stages T1c to T2b and 78.5% for patients in clinical stage T2c or greater ($P = 0.035$). (**b**) Kaplan–Meier estimate of PSA-free survival according to pathological stage. The 3-year PSA-free survival rate was 91.5% in patients who achieved pathological T0 or T2 status and 68.9% in those with T3 status ($P = 0.001$). (**c**) Kaplan–Meier estimate of PSA-free survival according to surgical margin status. The 3-year PSA-free survival rate was 86.9% in patients with negative surgical margins and 53.0% in those with positive surgical margins ($P < 0.0001$).

Table 2. Most common treatment-related toxicities

	No. (%)	
	Any grade	*Grade 3, 4*
Gynecomastia	140 (98.6)	0
Nausea	65 (45.8)	0
Anemia	15 (10.6)	0
Elevated AST	1 (0.7)	0

Abbreviation: AST, aspartate aminotransferase.

docetaxel.[22] Neoadjuvant ketoconazole and docetaxel chemotherapy has been reported to achieve 36% of PSA-free survival,[23] which showed no remarkable improvement compared with the previous literatures.[1,2] Furthermore, majority of the reported clinical trials in neoadjuvant setting were small-sized with relatively short follow-up. Most recently, at 2012 ASCO annual meeting, Taplin *et al.*[24] reported that neoadjuvant LHRH plus abiraterone acetate therapy achieved high pT0 rate (10%). The biochemical outcome was not presented, however, this regimen may be a promising for the treatment of high-risk Pca.

The advantages of EMP as compared with other cytotoxic drugs are its estrogenic effects[25] as well as its ease of oral administration and relatively good tolerability at the effective dose. However, in our preliminary study, a number of patients experienced gastrointestinal adverse effects at regular dose with 560 mg day^{-1}.[17] Therefore, we reduced the dose of EMP to 280 mg day^{-1} for continuous administration for 6 months.

An LHRH agonist administration desensitizes the pituitary and reduces testicular function. However, switching of an LHRH agonist to other LHRH agonist in castration-resistant Pca patients achieved further decrease in PSA levels.[26] This event suggests that LHRH agonists may have other important functions such as directly effect on testicular tissue function.[26] Furthermore, several studies have shown that LHRH agonist treatment directly inhibits the proliferation of both androgen-sensitive and androgen-insensitive Pca with no relation to androgen levels.[27–29] EMP decreases serum testosterone to castration level,[25] however, there is an advantage to use EMP in combination with LHRH agonist. In fact some authors reported the superiority of combination of EMP and LHRH agonist to orchiectomy alone or androgen deprivation therapy for the patients with advanced Pca.[15,16,30]

In this study, 4.9% of patients achieved pT0, which can be acceptable antitumor effect. No serious adverse events occurred and there were no toxicity-related deaths. All patients completed the protocol therapy and underwent RP without delay. We experienced 1 (0.7%) rectal injury, leakage from the vesicourethral anastomosis in 26 patients (18.3%) and surgical site infection occurred in 3 (2.1%), which are acceptable as complications involved in RP for patients with high-risk Pca.

The long-term administration of low-dose EMP may have positive impact on the relatively long PSA-free survival of 84.3%, which corresponds to that in low- or intermediate-risk patients who treated RP alone in our clinic.[18]

However, our study has some limitations including study design and participant population. This study is a single-arm trial performed at a single institution in Japan. The mean follow-up period of 34.9 months is not long enough and all participants were Japanese. Several authors have indicated the racial differences in response and adverse effect of hormonal therapy in PCa.[31,32] These issues should be addressed in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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