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Stat3 Orchestrates Tumor Development and Progression: The Achilles' Heel of Head and Neck Cancers?

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Abstract: Despite recent advancements in treatment modalities, the overall survival and quality of life of patients with head and neck squamous cell carcinoma (HNSCC) have not significantly improved over the past decade. With the increasing emergency of new biological agents, the development of novel treatment schemes based on cancer cell biology may be promising for this group of patients. We previously introduced the "oncogene addiction" concept as a rationale for molecular targeting in cancer therapy and prevention. In this context, an increasing number of preclinical studies have demonstrated that the Signal Transducers and Activators of transcription 3 (Stat3) transcription factor play critical roles in the development and progression of a variety of tumors including HNSCC, by regulating cell proliferation, cell cycle progression, apoptosis, angiogenesis, immune evasion, Epithelial-Mesenchymal Transition (EMT) and through effects in cancer stem cells. The purpose of this review is to summarize current experimental and clinical evidence that suggests that HNSCC might be addicted to Stat3 and describes the molecular mechanisms that may explain this phenomenon. In addition, we discuss whether this addiction is an exploitable target for developing approaches for the treatment and prevention of HNSCC.

Keywords: Head and neck cancer, Stat3, molecular targeting, oncogene addiction, small molecule inhibitor.

INTRODUCTION

Multistage Carcinogenesis and the Concept of Oncogene Addiction in HNSCC

Head and neck squamous cell carcinoma (HNSCC) arises in the upper aerodigestive tract and is the sixth most common cancer worldwide [1]. Despite many recent advances in the treatment of cancers, the overall survival of patients with HNSCC has not significantly improved over the past decade [1]. In addition, treatment for patients with advanced stage disease remains largely dependent on radical surgery which often compromises major organs including the larynx, tongue, and pharynx, leading to substantial impairments in quality of life (QOL). The development and progression of HNSCC occur through a stepwise and progressive accumulation of genetic and epigenetic alterations mainly due to direct and repeated exposures to carcinogens including tobacco, alcohol, and viral infections [1-3]. Thus, HNSSC is a classical model of multistep carcinogenesis and field carcinogenesis, a concept originally proposed by Slaughter [4], that described a series of multifocal lesions within the same field [1-3]. In this genomic era, the design of novel treatment approaches that target these alterations may prove effective in the treatment and prevention of HNSCC.

During multistage carcinogenesis, the progressive accumulation of genetic alterations often leads to an extensive disruption in the genomic circuitry of the cell. However, despite the development of this "bizarre" circuitry, these cells remain addicted to the continued activity of only one or a few specific oncogenes for the maintenance of the malignant phenotype [5-7]. We have described this phenomenon as "oncogene addiction", and there are increasing number of preclinical and clinical reports that illustrate this concept, where the reversal of only one or a few of these abnormalities can profoundly inhibit the growth of cancer cells and in some cases lead to improved survival rates [5-7]. Thus, oncogene addiction supports the rationale for developing molecular targeting approaches in both cancer therapy and prevention. Related reviews based on this concept have recently been published [8, 9]. To date, the critical onogene (i.e., Achilles' heel) of HNSCC has not yet been identified.

Increasing number of studies have demonstrated that the Signal Transducers and Activators of transcription 3 (Stat3) transcription factor play significant roles in the development and progression of a variety of cancers including HNSCC, by regulating cell proliferation, cell cycle progression, cell survival (i.e., inhibition of apoptosis), angiogenesis, immune evasion and epithelial-mesenchymal transition (EMT) [10, 11]. Stat3 appears to be a critical molecule for the maintenance of the cancer phenotype in HNSCC, and therefore may represent the candidate oncogenes to which HNSCC is addicted. The purpose of this review is to summarize the current experimental and clinical evidence that supports the concept that HNSCC might be addicted to Stat3 signaling, and describe the molecular mechanisms that may explain this phenomenon. In addition, we discuss whether this addiction is an exploitable target for the prevention and treatment of HNSCC.

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EVIDENCE FOR STAT3 ADDICTION

Signal transducers and activators of transcriptions (STATs) comprise a family of cytoplasmic transcription factors (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6) that were first identified as the key mediators of cytokine signaling by Darnell *et al.* in 1991 [12]. In normal cells, the activation duration of Stat3 in response to growth factors is transient and usually lasts from a few minutes to several hours [13]. Constitutive activation of Stat3 was first described in transformed cells as a consequence of the oncogenic tyrosine kinase v-Src. In 1996, Darnell and his colleagues demonstrated that introduction of a constitutively active mutant form of Stat3 into fibroblasts induced cellular transformation and these transformed cells were able to form tumors in nude mice, thus providing evidence that Stat3 is an oncogene [14]. Following this report, constitutive activation of Stat3 has been demonstrated in a variety of human cancer cell lines and clinical samples, and the multiple roles of this transcription factor in tumorigenesis and tumor progression have been intensively studied in rapidly expanding literature (for review, see ref. [11]). In HNSCC, constitutive activation of Stat3 was first demonstrated by Grandis *et al.* [15, 16]. They found that activation occurs early during HNSCC carcinogenesis due to the autocrine activation of TGF-α/EGFR signaling [15, 16], which is observed in a majority (80-100%) of HNSCC. [17-19]. We and other investigators have also demonstrated constitutive activation of Stat3 in primary HNSCC tissue specimens using immunohistochemistry or protein micro-arrays [20-23]. In addition to Stat3, activation of tumor suppressive Stat1 and oncogenic Stat5 has also been reported in HNSCC [11], but the roles of these two proteins are beyond the scope of this manuscript. Transfection of a constitutively active Stat3 construct in HNSCC cells resulted in increased proliferation *in vitro* and higher rates of *in vivo* tumor growth in mice [24], while inhibition of Stat3 using a dominant negative Stat3 construct, antisense oligonucleotides, a transcription factor decoy or siRNA led to decreased saturation density, increased serum dependence, growth inhibition and induction of apoptosis [15, 16, 20, 25-27]. In a xenograft model of HNSCC, administration of a Stat3 decoy or siRNA resulted in decreased tumor volume and induction of apoptosis [25, 26]. We and others [20, 22] found that Stat3 activation was significantly correlated with poor prognosis in the patients with squamous cell carcinoma of oral cavity. Taken together, these findings provide both laboratory and clinical evidence that HNSCC is significantly dependent on Stat3 signaling for the maintenance of the malignant phenotype, and thus might be addicted to Stat3.

MECHANISMS OF STAT3 ADDICTION (FIG. 1)

Diversity of Stat3 Activating Pathways in HNSCC

Activation of Stat3 signaling can occur through diverse nonspecific and HNSCC-associated pathways. As described above, Grandis *et al.* first reported that the frequent Stat3 activation in HNSCC occurs as a consequence of autocrine activation of TGF-α/EGFR signaling [15, 16]. In recent studies, Gutkind and his colleagues demonstrated that in a subset of HNSCC, autocrine activation and/or paracrine stimulation by IL-6 of the gp130/Jak pathway is primarily responsible for Stat3 activation rather than EGFR activation [28, 29]. In addition, they found that enhanced IL-6 secretion in HNSCC is dependent on NF-κB activity, suggesting the existence of cross-talk between the NF-κB and Stat3 signaling systems [29]. A recent study by Lee *et al.* also supports the relevance of IL-6 signaling in Stat3 activation in HNSCC [30]. In this study, hypermethylation (i.e., transcriptional silencing) of the Jak inhibitor, SOCS, and subsequent enhanced activation of Stat3 by IL-6 and gp130 were observed in about one third of HNSCC [30]. The non-receptor tyrosine kinase, Src, also plays a role in the activation of Stat3 in response to TGF-α stimulation [31]. Of note, Stat3 activation by TGF-α/EGFR, IL-6/gp-130/Jak or Src is not specific for the development of HNSCC, and a recent comprehensive review describes the interactions of these molecules in more detail [11].

Several investigators have recently identified Stat3 activating pathways, which appear to be highly associated with HNSCC carcinogenesis [21, 32, 33]. Arredondo *et al.* found that in oral keratinocytes, nicotine, a major carcinogen in tobacco, can activate Stat3 through the alpha 7 nicotinic acetylcholine receptor [32]. This tobacco-related Stat3 activation, at least in part, explains the markedly high rate of Stat3 activation (>80%) in clinical samples of oral squamous cell carcinoma that develop in tobacco-chewers [21]. It is now apparent that latent Epstein-Barr Virus (EBV) infection plays a critical role in the development of nasopharyngeal carcinoma, that displays unique biological properties among the HNSCCs [34]. Intriguingly, EBV infection of nasopharyngeal epithelial cells *in vitro* promoted activation of Stat3 and its downstream targets, providing the first report that Stat3 may mediate EBV-induced carcinogenesis in nasopharyngeal carcinoma [33]. Thus, it appears that constitutive activation of Stat3 in HNSCC can occur through diverse nonspecific and HNSCC-associated Stat3 activating pathways. The convergence of these diverse pathways on Stat3 activation suggests that Stat3 may be the oncogene to which HNSCC is addicted. A study by Kijima *et al.* further provides evidence that HNSCC may be addicted to Stat3 activation [24]. HNSCC cells that were transfected with a constitutively active Stat3 construct were no longer sensitive to either the growth enhancing effects of TGF-α or the growth inhibitory effects of an EGFR inhibitor [24]. This suggests that when Stat3 is constitutively and sufficiently activated by an alternative mechanism, the growth of these cells is no longer dependent on extrinsic Stat3 activating pathways. In other words, activation of Stat3, not the activation of EGFR, is the crucial driving force for cell proliferation. Therefore, the multiple and diverse Stat3 activating pathways appear to provide a safeguard for Stat3 activation, in case where one or a few pathways are lost with the genetic or epigenetic events that occur during tumor progression. In this context, direct inhibition and targeting of Stat3 will be a far more rational and powerful method for cancer prevention and therapy, rather than indirect inhibition of the multiple upstream Stat3 activating molecules (e.g., EGFR, Jak, Src). This aspect is discussed further in the targeting Stat3 section.

Effects of Stat3 Activation on the Expression of Cyclin D1 and c-myc

Over the past decade, it has become apparent that carcinogenesis is frequently associated with mutations or

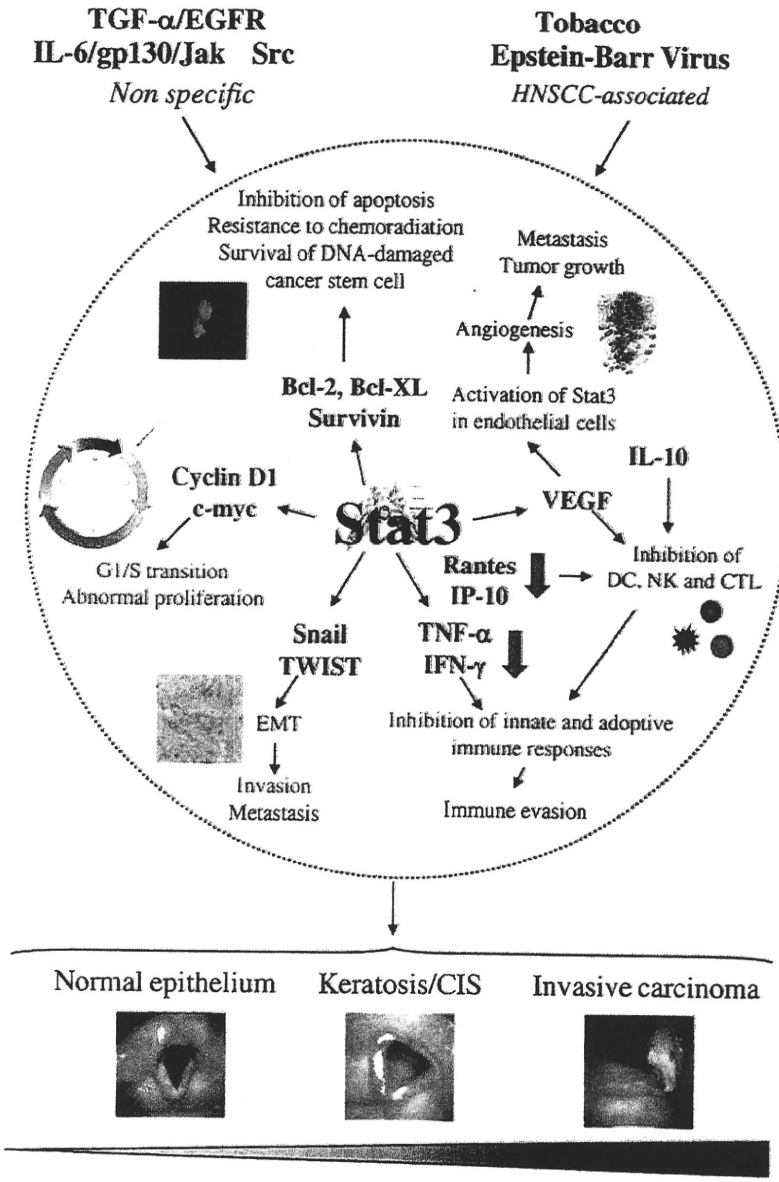

Fig. (1). A proposed mechanism by which HNSCC is addicted to Stat3.

Constitutive activation of Stat3 in HNSCC is caused by diverse signal transduction pathways. The frequent activation of TGF-α/EGFR in HNSCC was believed to be a major Stat3 activating pathway. However recent studies demonstrated that IL-6/gp130/Jak preferentially activates Stat3 in a subset of HNSCC. Src also plays causative roles in Stat3 activation. These Stat3 activating pathways are not HSCC-specific. On the other hand, recent findings displayed Stat3 activating mechanisms that are closely associated with HNSCC carcinogenesis. Tobacco or EBV latent infection activates Stat3 in oral keratinocyte or in nasopharyngeal epithelial cells. Stat3 transcriptionaly upregulates the levels of cyclinD1 and c-myc proteins, which result in the accerelated G1/S transition and abnormal proliferation. Anti-apoptotic proteins, Bcl-2, Bc-XL and survivin are also targets of Stat3. Overexpressions of these proteins inhibit apoptosis, promote cell survival and enhance cellular resistance for chemoradiation. Furthermore, it is proposed that these ani-apoptotic proteins protect DNA-damaged cancer stem cells from elimination by apoptosis and thereby allow them to expand clonally. In tumor cells, activated Stat3 enhances VEGF production and secretion. This VEGF, in turn activates Sat3 in endothelial cells, which is required for endothelial cell migration and therefore vessel formation. Consequent angiogenesis promotes tumor growth and metastasis. Stat3 is a negative regulator of both innate and adoptive immune responses. Tumor cells expressing constitutively active Stat3 produce markedly decreased levels of inflammatory cytokines, TNF-α, IFN-γ, RANTES and IP-10 and thereby inhibit both acute and adoptive immune responses. In addition, these tumor cells produce significantly increased levels of immunosuppressive cytokines and growth factors (e.g. VEGF and IL-10), which inhibit the functions of DCs, NK cells and CTLs. As a result, tumor cells with constitutive active Stat3 develop the state of "immune evasion". Recent findings suggest that Stat3 is involved in the process of EMT, at least in part, by activating Snail and TWIST. Tumor cells undergoing EMT acquire the ability to migrate and metastasize. Taken together, these findings indicate that Stat3 dominates (i.e. "orchestrates") the entire process of tumor development and progression of HNSCC.

abnormalities in the expression of various cyclins, CDKs and CDIs in a variety of human cancers including HNSCC [5]. In particular, the role of cyclin D1, which acts at the mid-portion of the G1-S transition, has been intensively studied in HNSCC [2, 35]. We and many others have demonstrated that the overexpression of cyclin D1 occurs in over 50 % of the cases of HNSCC and is a marker of poor prognosis in this disease [2, 35]. However, the cyclin D1 gene is amplified in only about 20% of the cases [2], and the mechanism of cyclin D1 overexpression is unclear in a majority of these cases. We previously found that the inhibition of Stat3 activity by a dominant negative Stat3 construct decreased cellular levels of cyclin D1 expression at the level of transcription [20]. In addition, in clinical samples of oral squamous cell carcinoma, there was a significant correlation between Stat3 activity and increased cyclin D1 expression [20]. These results provided the first evidence that in HNSCC constitutive activation of Stat3 may play a causative role in the overexpression of cyclin D1. This association was confirmed in several recent *in vitro* and *in vivo* studies [24, 26, 36, 37]. In addition, the significance of cyclin D1 in Stat3-dependent tumorigenesis was discovered in a recent study by Leslie *et al.* [38]. They found that cyclin D1 is required for Stat3-induced transformation (anchorage-independent growth) of immortalized fibroblasts. These findings, taken together with the fact that both the overexpression of cyclin D1 and the constitutive activation of Stat3 occur during relatively early phases of HNSCC carcinogenesis, suggest that cyclin D1 is a critical downstream target in the process of initial tumor development caused by Stat3.

The *c-myc* proto-oncogene encodes the c-myc transcription factor, and elevated or deregulated expression of c-myc has been detected in a wide variety of human cancers including HNSCC [39-41]. Overexpression of c-myc is associated with aggressive and poorly differentiated tumors and is therefore a marker of poor prognosis in patients with HNSCC [40, 41]. Although the precise mechanism of c-myc overexpression in HNSCC is not clear, it is now apparent that c-myc can be transactivated by Stat3 [11]. Indeed, EBV infection in nasopharyngeal epithelium can elevate the expression level of c-myc in a Stat3-dependent manner [33]. As seen in the pattern of cyclin D1 overexpression, c-myc overexpression also occurs during early stages of HNSCC carcinogenesis in an animal model of oral cancer [42] and in an *in vitro* transformation model of oral-esophageal cancer [43]. Thus, in HNSCC both c-myc and cyclin D1 appear to be crucial effecter proteins, that are active during the early phases of Stat3-induced tumorigenesis.

Effects of Stat3 Activation on Cell Survival (i.e. Inhibition of Apoptosis) and Sensitivity to Chemotherapy and Radiation Therapy

Sustained activation of Stat3 may lead to tumor promotion, in part, by upregulating the expression levels of two classes of anti-apoptotic protein family, the Bcl-2 family (e.g., Bcl-2, Bcl-XL and MCL-1) and the inhibitor of apoptosis (IAP) family (e.g., survivin) [11, 44]. Bcl-2 family proteins inhibit apoptosis by acting upstream of mitochondria prior to activation of caspases, while IAP family proteins exert their effects after caspase activation [44]. Deregulated expression of these anti-apoptotic proteins can protect cancer cells from apoptosis and allow them to survive during tumor progression [44]. In addition, overexpression of these proteins may prevent apoptosis following treatment with conventional DNA-damaging cancer therapies (i.e., chemotherapy and radiation), thus significantly decreasing cellular sensitivity of cancer cells to these therapies [44]. Indeed, both precursor and established lesions of HNSCC display frequent overexpression of Bcl-2, Bcl-XL or survivin and this overexpression is significantly associated with aggressive phenotype (i.e., advanced TNM stage) [45-48]. Overexpression of Bcl-2 or Bcl-XL is also correlated with poor prognosis in early stage HNSCC after treatment with radiation [48, 49]. However, it should be noted that other investigators reported a paradoxical association between increased Bcl-2 expression and favorable outcome in patients with HNSCC [47, 50]. Increased survivin expression was significantly associated with aggressive phenotype, presence of nodal and distant metastases, and poor clinical outcome in HNSCC [51-54]. The association between survivin and tumor metastases is consistent with a recent theory, which proposes that cancer stem cells are protected from apoptotic elimination by virtue of anti-apoptotic proteins and are thereby allowed to expand clonally until they acquire metastatic ability [55, 56]. This concept will be discussed in more detail in the Effects of Stat3 Activation and EMT and cancer stem cells section.

The important roles of Stat3 in regulating anti-apoptotic proteins have been demonstrated in both HNSCC cancer development and treatment. Stat3-induced expression of Bcl-XL was observed after both EBV-infection of nasopharyngeal epithelium and stable expression of constitutively activated Stat3 in HNSCC [24, 33]. Conversely, inhibition of Stat3 activity by a dominant negative Stat3 construct, antisense oligonucleotides, decoy oligonucleotides and siRNA led to decreased levels of Bcl-2, Bcl-XL and survivin, inhibition of tumor growth and induction of apoptosis [16, 20, 25-27, 36]. Treatments of laryngeal cancer cells with sulindac, a nonsteroidal anti-inflammatory drug induced apoptosis and growth inhibition *in vivo* due to decreased survivin expression caused by the downregulation of Stat3 [57]. We and other investigators found that inhibition of Stat3 markedly enhanced *in vitro* and *in vivo* sensitivity of HNSCC cells by down-regulating Bcl-2 and Bcl-XL expression to the chemotherapeutic agents, 5-fluorouracil and cisplatinum [36, 58]. It was also demonstrated that indirect inhibition of Stat3 by EGFR inhibitor can significantly promote sensitivity of HNSCC cells to radiation [59, 60].

Effects of Stat3 Activation on Angiogenesis

Angiogenesis, the recruitment of new capillaries from existing vessels, is indispensable during the processes of tumor development, three dimensional tumor growth, and metastasis of solid tumors including HNSCC [61-63]. To date, several angiogenic factors have been identified. Among them vascular endothelial growth factor (VEGF) is one of the most prominent mitogens for endothelial cells, and the role of VEGF in carcinogenesis has been extensively investigated [62, 64]. Elevated VEGF production has been associated with both higher levels of intratumoral microvessel density (IMVD) and poor prognosis in the patients with several ma-

lignancies including HNSCC [65-69]. The precise mechanism by which VEGF is overexpressed in HNSCC is not known. In our previous *in vitro* study, we found that Stat3 directly activates VEGF production *via* a putative Stat3 responsive element (-849 to -842) on the VEGF promoter [70]. Furthermore, in clinical samples of oral HNSCC, we found significant correlations between Stat3 activity, VEGF production, and angiogenesis [70]. These findings are consistent with a recent study by Xi *et al.* [36], according to that administration of a Stat3 decoy significantly decreased the expression level of VEGF and thereby inhibited tumor growth and angiogenesis in a xenograft model of HNSCC. Furthermore, in endothelial cells the activation of Stat3 signaling by VEGF is required for both migration and vessel formation [71]. Thus in HNSCC angiogenesis, Stat3 appears to have at least two different functions: Stat3 enhances VEGF production by HNSCC cells and this VEGF, in turn, promotes Stat3 activity in endothelial cells, required for angiogenesis.

Effects of Stat3 Activation on Immune Evasion

Previous studies suggest that tumors including HNSCC can escape from rejection by both innate and adoptive immune responses through "immune evasion" or " immune tolerance" [72, 73]. It has been shown that tumor "toleration" is an active process caused by tumor cells resulting from imbalances in the tumor microenvironment, including alterations in antigen-presenting-cell subsets (e.g., dendritic cells (DC)), in co-stimulatory and co-inhibitory molecules and in ratios of effecter (e.g., NK and CTL) and regulatory T cells [72, 73]. Interestingly, Stat3 may negatively regulate these inflammatory responses. Mice whose macrophages and neutrophils are devoid of the Stat3 gene develop chronic enteritis, and $Stat3^{-/-}$ macrophages can restore the responsiveness of tolerant CD^{4+} T cells and reverse systemic tolerance [74, 75]. Recent studies have demonstrated that constitutive activation of Stat3 in both tumor cells and components of the host immune system may cooperate to impair both innate and adoptive immune responses to tumors [76-78]. Tumor cells expressing constitutively active Stat3 produce markedly decreased levels of TNF-α, IFN-γ, RANTES and IP-10, which results in the inhibition of acute inflammatory responses and decreased migration and infiltration of neutrophils, macrophages, and T-cells into the tumor site [73, 77, 78]. These tumor cells also produce increased levels of immunosuppressive cytokines and growth factors (e.g. VEGF and IL-10) [73, 78], that, in turn, activate Stat3 in immature DCs, and block DC differentiation [76, 78]. The subsequent lack of mature antigen-presenting DCs results in T-cell tolerance to tumor antigens [73, 78]. Taken together, tumor cells with constitutive activation of Stat3 exert multiple effects on the host immune system that lead to immune evasion and the subsequent development of human cancers, including HNSCC. The roles of Stat3 in HNSCC immune evasion are comprehensively described in a recent review [73].

Effects of Stat3 Activation on EMT and Cancer Stem Cell

At the final step of malignant tumor progression, epithelial tumors including HNSCC acquire the ability to metastasize distant organs - this is often accompanied by a process known as Epithelial-Mesenchymal Transition (EMT) during which cancer cells change from a highly differentiated, epithelial morphology to a migratory invasive phenotype [79, 80]. During EMT, epithelial cancer cells undergo down-regulation of apical and basolateral adherent molecules including E-cadherin and cytokeratins and upregulation of mesenchymal molecules including vimentin and N-cadherin, thereby losing cell-cell contact and gain the cell motility [79, 80]. It was recently shown that Stat3 activation is essential for EMT in the zebrafish gastrula organizer [81]. In this system, Stat3 upregulated a zinc transporter protein, LIV-1, which functions as a nuclear transporter of the zinc-finger protein Snail, a master regulator of EMT [81]. Yang *et al.* found that Stat3 activation plays an important role in TGF-β-mediated EMT [82], while Lo *et al.* showed that Stat3 mediates EGF/EGFR-induced EMT by upregulating the expression of TWIST [83]. The results of these studies strongly suggest that Stat3 may be an important regulator of EMT and tumor progression. Of note, Stat3 activation and EMT have not been studied in HNSCC yet.

As described in a recent review, the concept of EMT and the cancer stem cell theory contribute importantly to distinct features of carcinogenesis - the existence of heterogeneous cell populations in a single tumor and the dynamically changing phenotypes displayed by individual cancer cells during EMT or MET [56]. Thus, in addition to the diverse functions of Stat3, that we have described in this review, we hypothesize that Stat3 might also be a master regulator of cancer stem cell development in HNSCC. A recent study of skin carcinogenesis provided a striking example that may confirm this hypothesis. In this model, the investigators demonstrated that Stat3 is required for the *de novo* epithelial carcinogenesis and maintained the survival of DNA-damaged stem cells. By upregulating Bcl-XL, cyclin D1 and c-myc, Stat3 induced the cell proliferation required for clonal expansion of initiated cells during tumor promotion [55]. This finding is not surprising when we think of the fundamental roles of Stat3 in normal cell physiology. During gastrulation, organogenesis, and wound healing, this latent cytoplasmic transcription factor is transiently activated in embryonic precursor cells or in normal somatic stem cells only during specific events, when this activation is required [84-86]. Thus, if stat3 was abnormally and constitutively activated in normal somatic stem cells in the upper aerodigestive epithelium, this activation could lead to the development of cancer stem cells in this tissue, as seen with the abnormal expression of β-catenin during colon carcinogenesis [56, 79, 87].

In summary, it is quite surprising that activation of a single transcription factor is profoundly associated with the diverse steps that are critical for the development and progression of HNSCC. These findings strongly suggest that HNSCC is addicted to the activation of Stat3, and therefore, this activation might be the Achilles' heel of HNSCC. A proposed mechanism by which HNSCC is addicted to Stat3 is summarized in Fig. (1).

TARGETING STAT3

A successful and feasible molecular targeting approach must fulfill several criteria: (1) the particular cancer is highly dependent on (i.e., addicted to) the targeted molecule, (2) the

potential for escape from the given state of oncogene addiction is low, (3) strategies for targeting the particular oncogene are clinically available, and (4) treatment with the targeted agent is associated with minimal or tolerable toxicity. The evidence for Stat3 addiction in HNSCC (criterion 1) has been discussed in the preceding sections. In this section, we will discuss whether Stat3 targeting can fulfill the remaining criteria.

Escape from the State of Oncogene Addiction

As already described in our previous review, cancers can potentially escape from a given state of oncogene addiction [7]. For example, in the Her-2/neu breast cancer model, tumors that recurred after treatment with trastuzumab were found to be Her-2/neu independent. These tumors contained increased expression of the transcription factor Snail, suggesting that recurrent tumors escaped from the addiction to Her-2/neu and were secondary addicted to Snail [7]. However, this finding may also suggest that the activation of Her-2/neu is required during the early phase but not in the advanced phase (i.e., recurrence) of tumor development; for Snail the converse would be true. Thus, activation of these two genes may play critical and transient roles during particular stages of breast tumor development and progression. In contrast, as we have discussed in this review (Fig. 1), the activation of Stat3 appears to "orchestrate" the entire process of HNSCC development and progression. Therefore, it would be quite difficult for HNSCC to completely escape from the addiction to Stat3. On the other hand, HNSCC could escape from the influences of Stat3 targeted downstream genes (i.e., cyclin D1, Bcl-XL or VEGF) during particular stages of carcinogenesis. In this model, the potential for escape from Stat3 addiction would be low (criteria 2). However, it should be noted that activation of the transcription factor NF-κB might provide an additional target in HNSCC, since these two transcription factors transactivate a surprisingly overlapping set of downstream molecules (e.g., c-myc, cyclin D1, Bcl family proteins, IAPs and VEGF) in HNSCC, as summarized in our recent study [88]. Theoretically, combined inhibition or targeting of Stat3 and NF-κB might exert synergistic activity against HNSCC. This possibility remains to be analyzed in future *in vitro* and *in vivo* studies.

Strategies for Targeting Stat3

A considerable number of strategies can be effective for the inhibition or targeting Stat3. These strategies are generally divided into three categories based on their mechanisms of action: (1) indirect stat3 inhibition following the inhibition of Stat3 activating molecules (e.g., EGFR, Jak and Src) or the activation of Stat3 inhibiting molecules (e.g., SOCS and IAST), (2) direct inhibition of Stat3, and (3) unknown or non-specific mechanisms (i.e., mechanisms of action are not clearly elucidated or difficult to categorize). These strategies including particular compounds have been described in several recent reviews [11, 89-91]. Cucurbitacin I, a selective Jak inhibitor, and indirubin, a Chinese herb constituent, can indirectly inhibit Stat3 by inhibiting the kinase activity of Jak or Src [92, 93]. We previously demonstrated that the green tea compound EGCG can inhibit the activation of Stat3 in HNSCC by inhibiting TGF-α/EGFR/erbB2 signaling, thus, providing another example of indirect Stat3 targeting [94]. On the other hand, direct inhibition of Stat3 may provide a more powerful and efficient strategy of Stat3 inhibition, since Stat3 activity can be maintained by multiple and diverse activating pathways as described in the Diversity of Stat3 activating pathways in HNSCC section. Until recently, methods for direct inhibition were limited mainly to genetic approaches using dominant negative constructs, antisense and decoy oligonucleotides or siRNA. Although these methods of "gene therapy" are powerful and reliable tools for *in vitro* or animal studies, their potential for clinical use remain much less clear. By contrast, the recent clinical success of using small molecule inhibitors like imatinib, which directly targets the BCR-ABL protein in CML, strongly supports the great potential for using molecular targeting drugs in cancer therapy [95]. In this context, investigators have recently discovered direct small molecule Stat3 inhibitors, STA-21, Stattic, and 531-201, which directly bind to the SH-2 domain of Stat3 and thereby inhibit Stat3 dimerization. These agents were identified using powerful structure-based virtual screening or high-throughput screening and fluorescence polarization assays [96-98]. These compounds strongly inhibited the growth of and induced apoptosis in breast carcinoma cells, which displayed constitutive activation of Stat3, while sparing normal fibroblast or carcinoma cells in which Stat3 is not activated [96-98]. Furthermore, S31-201 inhibited the growth of human breast carcinomas *in vivo* [98]. These studies provide support for the design of future Phase 1 clinical studies and strategies for the clinical targeting of Stat3 (criterion 3).

Possible Toxicity with Stat3 Inhibition

It is well known that Stat3 activation is required during early development, since Stat3-null mice display an embryonic-lethal phenotype [11]. On the other hand, non-tumor cells can proliferate and survive *in vitro* and *in vivo* following tissue specific knock-out of Stat3 [11]. In addition, numerous experimental studies demonstrated that growth inhibition or apoptosis caused by the inhibition of Stat3 is observed specifically in cancer cells, which display constitutively active Stat3, while sparing normal and cancer cells that do not overexpress Stat3 [11]. These findings might suggest that clinical therapy with Stat3 inhibitors may not be associated with serious systemic toxicity. However, in view of the fact that Stat3 is a ubiquitous transcription factor, this expectation is likely optimistic. Recent advancements in drug delivery systems, in particular, those based on nanomedicine, might be of great importance in reducing possible toxicities associated with Stat3 targeting, by allowing tumor-specific delivery of a small molecule Stat3 inhibitor [99]. Thus, these approaches may be used to develop agents that specifically target Stat3-addicted cancers while sparing normal, non-addicted tissue (criterion 4).

CONCLUSIONS AND FUTURE DIRECTIONS

The diverse functions of Stat3 appear to be essential in a variety of processes, which are highly associated with tumor development and progression of HNSCC. In addition, the recent discovery of small molecule Stat3 inhibitors might

soon provide the practical tools for targeting Stat3 clinically. Thus, the setting is ideal for validating clinical trials whether Stat3 is the long sought Achilles' heel of HNSCC.

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ABBREVIATIONS

CML	=	chronic myeloid leukemia.
CTL	=	cytotoxic T lymphocyte
DC	=	dendritic cell
EGFR	=	epidermal growth factor
EMT	=	epithelial mesenchymal transition
HNSCC	=	head and neck squamous cell carcinoma
IAP	=	inhibitor of apoptosis
IFN	=	interferon
IL	=	interleukin
IP-10	=	interferon induced protein 10
Jak	=	Janus kinase
NF	=	nuclear factor
NK	=	natural killer
RANTES	=	regulated on activation normal T cell expressed and secreted
SH2	=	src-homology 2
siRNA	=	small interfering RNA
SOCS	=	suppressor of cytokine signaling
STAT	=	signal transducer and activator of transcription
TGF	=	transforming growth factor
TNF	=	tumor necrosis factor
VEGF	=	vascular endothelial growth factor
v-src	=	viral-sarcoma

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ORIGINAL ARTICLE

Pharmaceutical interventions facilitate premedication and prevent opioid-induced constipation and emesis in cancer patients

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Abstract
Background Opioid analgesics possess a number of side effects, among which constipation and nausea/vomiting occur most frequently. Although pretreatment with laxatives and antiemetics for the prophylaxis of opioid-induced constipation and nausea/vomiting, respectively, is recommended, such side effects are still a matter of concern in clinical setting.
Methods We first surveyed the prevalence of premedication in 83 cancer patients who took opioid analgesics and the incidence of such side effects. Subsequently, intervention was carried out to promote premedication, and the effectiveness of the intervention was evaluated in 107 patients.
Results Prophylactic treatment with laxatives and antiemetics were conducted in 57% and 52%, respectively. The most frequently prescribed laxatives and antiemetics were magnesium oxide in combination with pantethine, a mild stimulant laxative, and prochlorperazine, respectively. The lack of premedication increased the risk of constipation (odds ratio, 5.25; 95% confidence intervals, 1.93–14.31; $p=0.001$) and vomiting (4.67, 1.04–21.04; $p=0.045$). Intervention such as provision of drug information to physicians, verification of prescription orders, and instructions to patients increased the rates of prophylactic medications to 93% ($p<0.001$) for laxatives and 81% ($p<0.001$) for antiemetics. The incidence of side effects was lowered from 36% to 9% ($p<0.001$) for constipation, from 28% to 17% for nausea ($p=0.077$), and from 16% to 4% for vomiting ($p=0.0085$).
Conclusion Intervention to promote prophylactic medication was highly effective in reducing the risk of opioid-induced constipation and nausea/vomiting.

Keywords Opioid analgesic · Constipation · Nausea/vomiting · Prophylaxis · Laxatives · Antiemetics

Introduction

A number of patients with advanced stage of cancers complain of pain that requires treatment with opioid analgesics [1–3]. Several preparations of strong opioid analgesics have been developed in recent years. Although opioid analgesics have a potent and effective antinociceptive action, the incidence of side effects sometimes leads to the reduction in the medication compliance and pain control [4–6]. In particular, constipation and nausea/vomiting are the most frequent side effects induced by opioid analgesics, since the minimal doses that induce constipation, nausea, and vomiting are even lower than those causing analgesia [7–9]. Opioid analgesics inhibit peristalsis of the small intestine through activation of opioid μ- and κ-receptors [10], which leads to constipation, while opioids cause nausea and vomiting through different mechanisms, including stimulation of the chemoreceptor trigger zone [11] and inhibition of gut motility [12]. Less common side effects are drowsiness, urinary retention, delirium, and respiratory depression [13–15]. Thus, several clinical practice guide-

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lines for cancer pain recommend the premedication with laxatives and antiemetics during therapy with opioid analgesics [16–18]. The duration of the treatment with antiemetics such as prochlorperazine is recommended to be about 1 week [19] because of an induction of tolerance to opioid-induced emesis [20]. In contrast, laxatives are suggested to prescribe continuously for the prevention of constipation [21], since the side effect prolongs as long as opioid analgesics are used [22].

In the first part of the present study, we retrospectively surveyed from medical records the prevalence of the prophylactic treatment with laxatives and antiemetics and the incidence of constipation and nausea/vomiting in cancer patients who took opioid analgesics in our hospital. Subsequently, we tried to promote prophylactic treatment by providing drug information to physicians, verifying prescription orders, and instructing to patients. Then, the effectiveness of such intervention on the incidence of opioid-induced constipation and nausea/vomiting was evaluated.

Patients and methods

Patients

The present study was carried out in accordance with the guidelines for the care for human study adopted by the ethics committee of the Gifu Graduate School of Medicine and notified by the Japanese government (approved no. 19-97 of the institutional review board). Eighty-three cancer patients who admitted to Gifu University Hospital and received opioid analgesics during January 2007–August 2007 were the subjects for the survey of the prevalence of premedication and the incidence of opioid-induced constipation and nausea/vomiting. Intervention was provided to 107 cancer patients who were admitted to our hospital during November 2007 to August 2008. Patients' characteristics are shown in Table 1. Patients with disease-modified constipation or nausea/vomiting and those who received antiemetics, including 5-HT_3 antagonists, for the prevention of chemotherapy-induced nausea/vomiting were excluded from the present study.

Survey of the prevalence of premedication in patients who took opioid analgesics

Constipation was defined as the lack of stools for more than 3 days in a week [23]. The number of days with stool in a week was also counted. Nausea and vomiting were graded according to the Common Terminology Criteria for Adverse Events v3.0 [24], and the incidence of grade ≥1 events was measured. Risk factors for the side effects induced by opioid analgesics were analyzed using multivariate logistic regression analysis.

Table 1 Patient characteristics before and after intervention

	Before intervention		After intervention		Statistical significance (p value)
	No. of patients	Percentage	No. of patients	Percentage	
Age (years)	66.1 (41–92)		63.7 (14–83)		0.424[a]
Total patients	83	107			
Male	43	51.8	67	62.6	0.177[b]
Female	40	48.4	40	37.4	
Type of cancer					
Lung cancer	19	22.9	23	21.5	0.957[b]
Gastroenterological cancer	42	50.6	51	47.7	0.798[b]
Gynecological cancer (uterine/ovarian)	6	7.2	5	4.7	0.664[b]
Head and neck cancer	6	7.2	7	6.5	1.00[b]
Urologic cancer	4	4.8	6	5.6	1.00[b]
Hematological cancer	1	1.2	7	6.5	0.146[b]
Others	5	6.0	8	7.5	0.917[b]
Opioid analgesic preparations taken					
Sustained-release tablet of oxycodone	64	77.1	87	81.3	0.596[b]
Sustained-release tablet of morphine sulfate	12	14.5	6	5.6	0.069[b]
Codeine phosphate	4	4.8	13	12.1	0.134[b]
Suppository of morphine hydrochloride	3	3.6	1	0.9	0.443[b]

[a] Mann–Whitney U test

[b] χ^2 test

Intervention

The following intervention was provided: (1) provision of drug information about side effects and prophylaxis of opioid analgesics to physicians on the basis of the clinical practice guidelines for cancer pain [16–18], (2) careful verification of prescription orders involving opioid analgesics, and (3) instruction to patients using a document. For patient education, we prepared a written form (A4 size) describing the incidence of adverse drug reactions associated with opioid analgesics and prevention or cure against such symptoms.

Statistical analyses

Data were analyzed using Statistics Program for Social Science for Windows (SPSS X, version 11, SPSS Incorporated, Chicago, IL, USA). Patients' characteristics before and after intervention were statistically compared by Mann–Whitney U test (age) or χ^2 test. A multivariate logistic regression analysis was carried out to determine odds ratio (OR) for opioid-induced constipation, nausea, and vomiting. The absence of premedication, gender, age over 65, and high daily doses (>20 mg) of opioid analgesics were analyzed as independent variables. The prevalence of premedication and the incidence of side effects were statistically compared before and after providing intervention by Fisher's exact probability test.

Results

Prevalence of premedication and the incidence of constipation and nausea/vomiting induced by opioid analgesics in cancer patients

Among 83 in-patients who took opioid analgesics, 47 patients (57%) were prescribed with laxatives concomitantly with opioid analgesics (Fig. 1a). The incidence of constipation was 56% in patients who did not receive premedication with laxatives, which was significantly (p=0.0024) higher than those who underwent prophylactic treatment (21%). The number of days with stool in 1 week was significantly (p=0.005) larger in the latter (5.6 days) than in the former

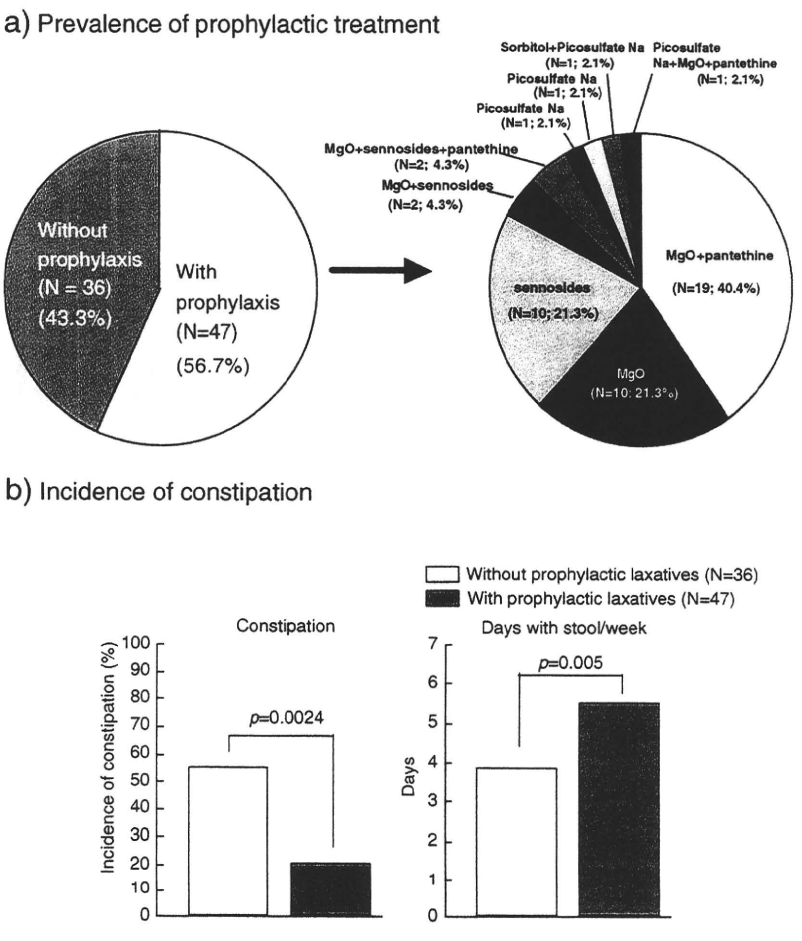

Fig. 1 Prevalence of the prophylactic treatment with laxatives in patients who took opioid analgesics (**a**) and the incidence rate of constipation in patients with or without prophylactic laxative treatment (**b**). Data were analyzed by Fisher's exact probability test

(3.9 days). Magnesium oxide and the colon-stimulating agent such as pantethine [25], a precursor of coenzyme A [26] with dyslipidemic action [27–29], and sennosides were commonly used as laxatives (Fig. 1a). The incidence of constipation decreased as the number of laxative preparations increased (Fig. 2a). In particular, the incidence of constipation was lowest in patients with prophylactic treatment with magnesium oxide in combination with pantethine (Fig. 2b). As shown in Fig. 2c, multivariate logistic regression analysis showed that the lack of prophylactic laxatives increased the risk of constipation compared to the presence of prophylactic laxative, in which the odds ratio was 5.25 (95% confidence intervals, 1.93–14.31, p=0.001).

On the other hand, 43 of 83 patients (52%) were prescribed with antiemetics for the prophylaxis of nausea and vomiting when they took opioid analgesics (Fig. 3a). The incidence of nausea and vomiting were 38% and 25%, respectively, in patients without prophylactic regimen; whereas the rates were 19% and 7%, respectively, in patients with prophylactic antiemetic agents (Fig. 3b). There was a significant (p=0.0339) difference in the rate of vomiting but not nausea between the two groups. The most commonly used antiemetic was the dopamine D_2 receptor blocker prochlorperazine (Fig. 3a). Other D_2 receptor blockers such as domperidone and metoclopramide were prescribed in a few patients (Fig. 3a). As shown in Fig. 4a, a multivariate logistic regression analysis revealed that female was at a significant risk of nausea (4.37, 1.42–13.47, p=0.01) and vomiting (17.57, 2.04–151.8, p=0.009). In addition, the lack of treatment with prophylactic antiemetics was a risk for vomiting (4.67, 1.04–21.0, p=0.045) but not for nausea (2.59, 0.88–7.62, p=0.08).

Evaluation of the effectiveness of intervention

Subsequently, intervention was provided to 107 patients who initiated therapy with opioid analgesics. There were no significant differences in the patients' characteristics, cancer types, and opioid analgesic preparations before and after interventions (Table 1). As shown in Figs. 5a and 6a, provision of intervention significantly enhanced the prevalence of the prophylaxis to 93% (100 of 107 patients, p<

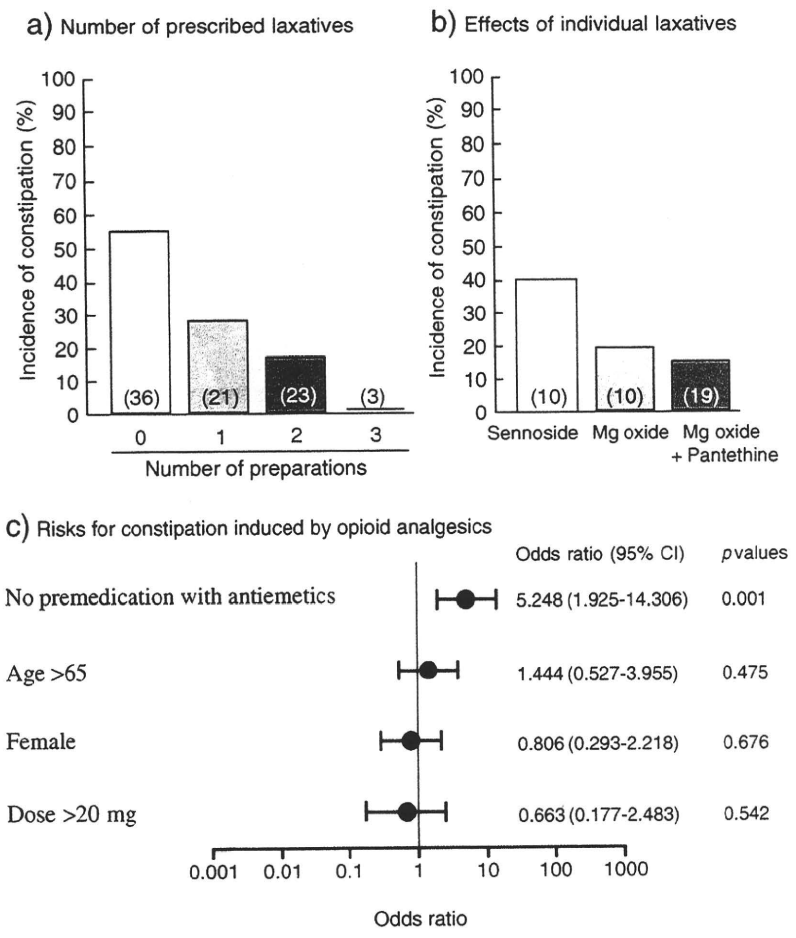

Fig. 2 Relationship between the incidence rate of constipation and the number of prescription of laxatives (**a**) or between the incidence of constipation and the individual laxatives (**b**) and forest plots of the odds ratio and 95% confidence intervals for several factors that affect the incidence of constipation associated with opioid analgesics (**c**). Figures in parentheses (**a** and **b**) represent the number of patients. In **c**, odds ratio was analyzed by multivariate logistic regression analysis

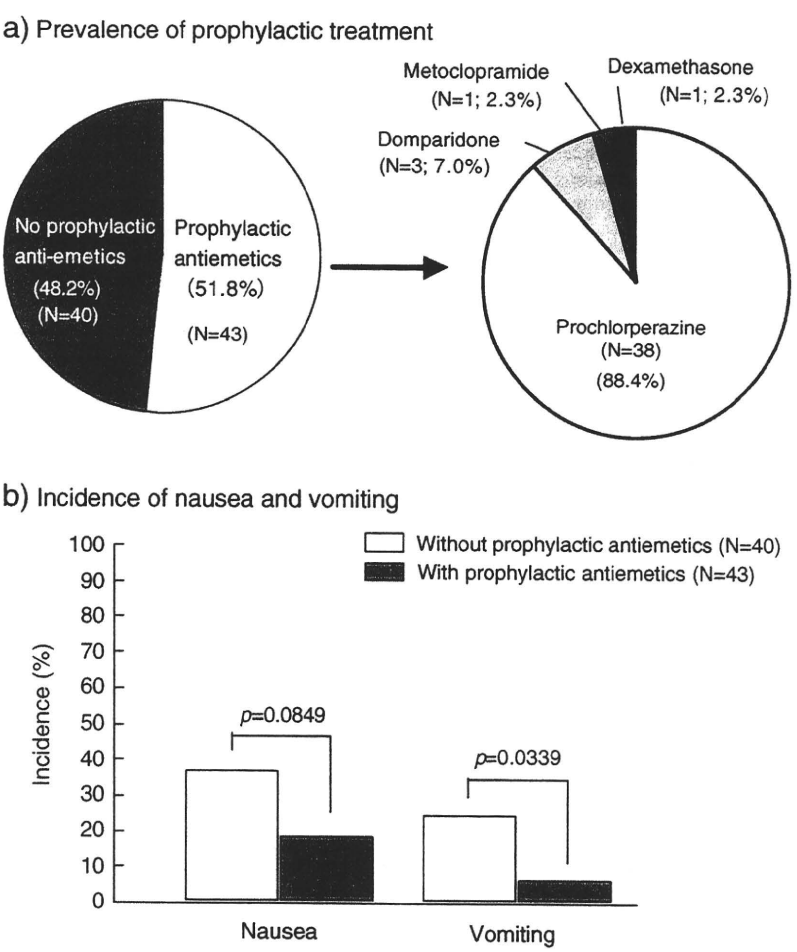

Fig. 3 Prevalence of prophylactic treatment with antiemetics in patients who took opioid analgesics (**a**) and the incidence rates of nausea and vomiting in patients with or without prophylactic antiemetic treatment (**b**). Data were analyzed by Fisher's exact probability test

0.001) for laxatives and 81% (87 of 107 patients, $p=0.001$) for antiemetics. Figure 5b shows the incidence of constipation before and after intervention. The incidence rate of constipation was significantly reduced from 36% (30 of 83 patients) to 9% (ten of 107 patients, $p<0.001$), thereby indicating that the risk reduction was 74% (relative risk, 0.259; 95% CI, 0.134–0.498).

On the other hand, as shown in Fig. 6b, the incidence rate of vomiting was significantly reduced from 16% (13 of 83 patients) to 4% (four of 107 patients, $p=0.0085$), although the rate of nausea was not significantly ($p=0.078$) lowered from 28% (23 of 83 patients) to 8% (18 of 107 patients).

Discussion

Morbidity associated with malignant carcinoma has increased in recent years. Most patients with end-stage advanced cancer suffer from intolerable pain that requires strong opioid analgesics, such as morphine, oxycodone, and fentanyl. The World Health Organization has published guidelines for improving the treatment of cancer pain worldwide, in which pain control is carried out according to the three-step analgesic ladder (from non-opioid analgesics through weak to strong opioids) depending on the pain intensity [30–32].

Opioid analgesics have various side effects, among which the most common side effects are constipation and nausea/vomiting. Opioid analgesics inhibit peristalsis of the small intestine through activation of opioid μ- and κ-receptors [10] at doses even lower than those required for antinociceptive action, indicating that constipation occurs in most cases in patients who take opioid analgesics. Without laxatives, opioid analgesics sometimes cause intractable constipation, coprostasis, and paralytic ileus, which may reduce the medication compliance, leading to poor control of pain. On the other hand, opioid analgesics elicit nausea and vomiting at doses that induce antinociceptive action, which may also cause a reduction in medication compliance. Several clinical practice guidelines, such as NCCN Clinical Practice Guideline in Oncology—Adult Cancer Pain 2009 [18] and the American Pain Society Guideline for Acute and Cancer Pain Management [17] have recommended the prophylactic treatment with laxatives and antiemetics.

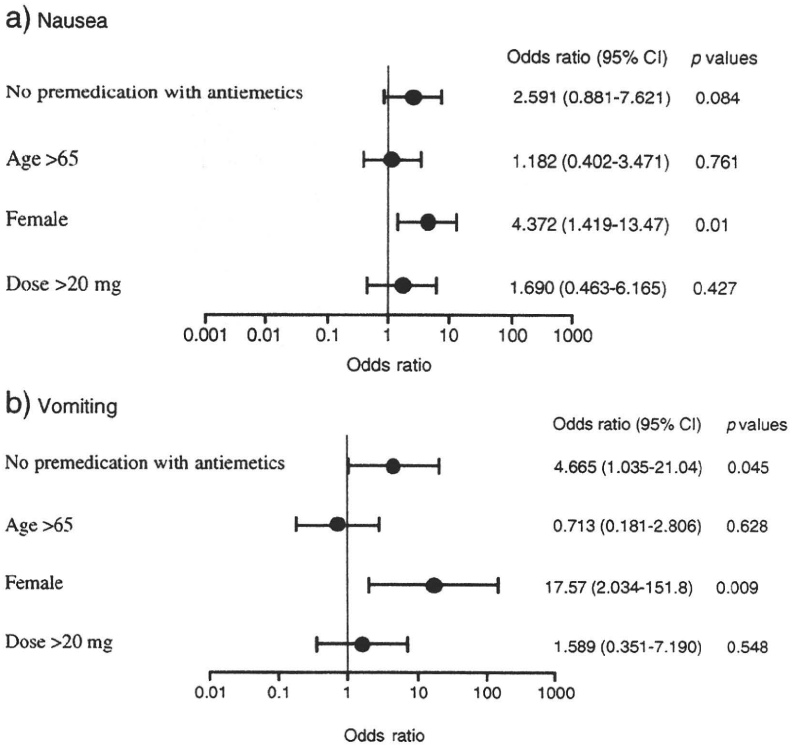

Fig. 4 Forest plots of the odds ratio and 95% confidence intervals for factors that affect the incidence of nausea (**a**) and vomiting (**b**) associated with opioid analgesics in patients who took opioid analgesics. Odds ratio was analyzed by multivariate logistic regression analysis

In the present study, 43% and 48% of patients who took strong opioid analgesics did not receive prophylactic treatment with laxatives and antiemetics, respectively. Constipation was significantly (p=0.002) more frequent in patients without premedication (56%) than in those with premedication (21%). The incidence of vomiting was significantly (p=0.034) higher in patients without prophylactic treatment (25%) than in patients with antiemetic treatment (7%). In the present study, drugs were handed directly to patients every time they should be taken, indicating a good compliance with drugs. Therefore, it is unlikely that the incidence of side effects in patients who prescribed with premedication is due to the low drug compliance, thereby suggesting that the conventional premedication with laxatives and antiemetics was not able to completely prevent opioid-induced constipation and emesis. The most frequently prescribed laxatives were magnesium oxide in combination with pantethine, a mild colon-stimulating laxative [25] with anti-hyperlipidemic action [27–29], while prochlorperazine was predominantly prescribed as the antiemetic agent. To effectively prevent the adverse reactions associated with opioid analgesics, we

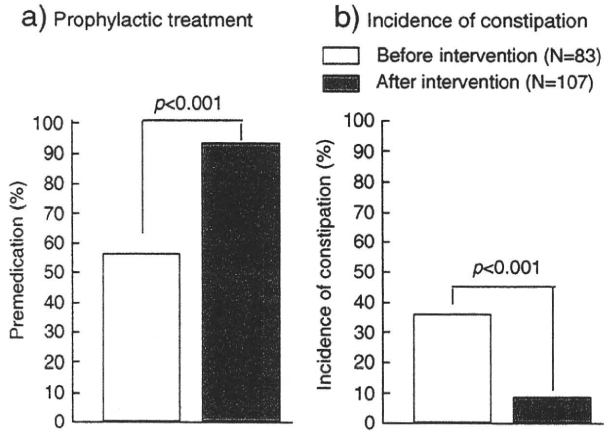

Fig. 5 Effect of intervention to facilitate prevalence of prophylactic medication on the incidence rate of constipation in patients who took opioid analgesics. Intervention was provided to 107 patients. Data were statistically evaluated by Fisher's exact probability test

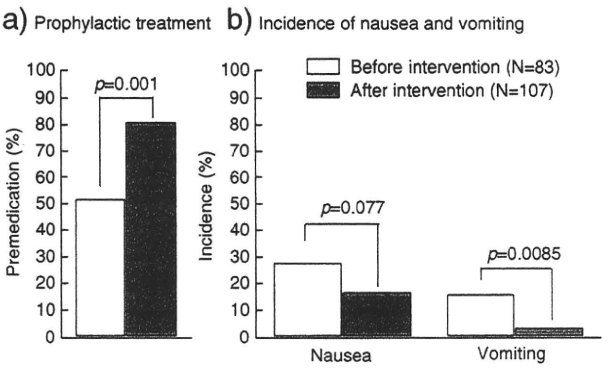

Fig. 6 Effect of intervention to facilitate prevalence of prophylactic medication on the incidence rates of nausea and vomiting in patients who took opioid analgesics. Intervention was provided to 107 patients. Data were evaluated by Fisher's exact probability test

carried out the following attempts to facilitate prescription of prophylactic regimens: provision of drug information about the side effects of strong opioid analgesics and the preventive measures, intensive verification of prescription orders involving strong opioid analgesics, and patient education. Such intervention was effective in reducing the incidence of constipation, nausea, and vomiting by promoting prescription of prophylactic laxatives and antiemetics. The incidence of constipation significantly reduced from 36% to 9%, while the incident rate of vomiting was significantly lowered from 16% to 4%, although such side effects were not completely prevented.

In conclusion, the prophylactic regimens for prevention of opioid-induced constipation and nausea/vomiting were not fully prevailed in our clinical setting. Several attempts, including provision of drug information about side effects and prophylaxis of opioid analgesics to physicians, verification of prescription orders involving opioid analgesics, and patient education using a document describing opioid-induced side effects and their prevention or cure, increased the prevalence of prescription for prophylaxis of opioid-induced side effects and ultimately led to the marked reduction in the incidence of constipation and nausea/vomiting. Therefore, laxatives and antiemetics should be prescribed in case opioid analgesics are prescribed.

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発がん物質の中期代替検索法

Alternative Animal Models for Carcinogenicity Testing – Evaluation of Gene-engineered Models –

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