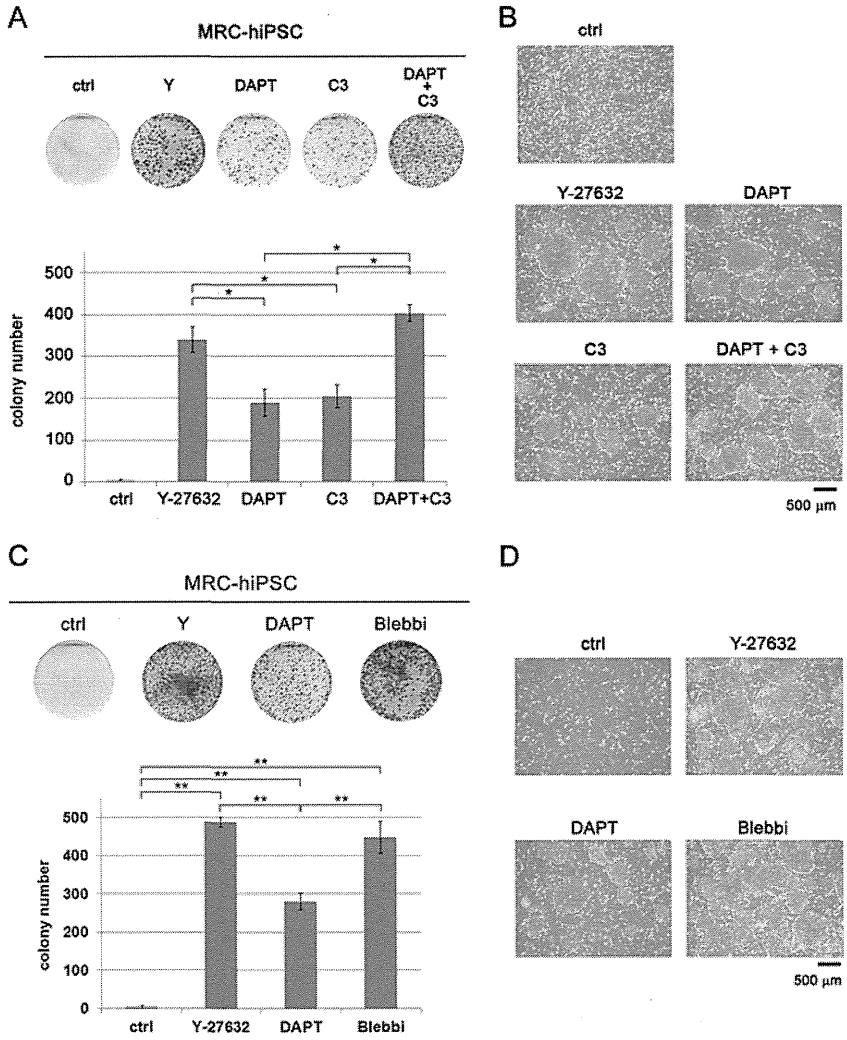

FIG 9 Activation of NOTCH and ROCK upon dissociation instigates loss of clonogenic growth capacity in hiPSCs. (A) MRC-hiPSCs, pretreated where indicated with 10 μM Y-27632 (Y) for 3 h, 10 μM DAPT for 3 h, 2.0 μg/ml C3 for 3 h, and 10 μM DAPT and 2.0 μg/ml C3 for 3 h, were completely dissociated by trypsin-EDTA treatment and pipetting. The dissociated cells were counted with Vi-CELL to confirm that the viabilities of each samples were greater than 90%, and aliquots of 2,000 cells were seeded on 60-mm dishes in the presence of MEF feeders. After 10 days of cultivation, the cells were stained with Giemsa's dye, and colonies were counted. The photographs are of representative dishes, and the graph illustrates means ± SDs. Treatment with any of these inhibitors significantly restored the clonal growth potential after dissociation. *, $P < 0.05$ according to Student's *t* tests. (B) Typical areas were photographed at 6 days postreplating. (C) MRC-hiPSCs, pretreated where indicated with 10 μM Y-27632 for 3 h, 10 μM DAPT for 3 h, and 10 μM blebbistatin for 3 h, were completely dissociated. The dissociated cells were seeded as for panel A, and colonies were counted. The photographs are of representative dishes, and the graph represents means ± SDs. **, $P < 0.01$ according to Student's *t* tests. (D) Typical areas were photographed at 6 days postreplating.

caspase-3 (Fig. 8C), implying a novel mechanism of ROCK inhibition by C3 independent of Rho inhibition. However, the mechanism underlying the inhibitory effect of C3 on NOTCH1 activation is currently unknown and awaits further investigation.

A recent report on the NOTCH1 nuclear interactome reveals ROCK1 as one of its interacting partners, raising the possibility of cross talk between these proteins (60). However, we have no evidence for interaction of the intracellular NOTCH1 proteins with caspase-3 and ROCK1 at present. Certainly, identification of a signaling mediator between NOTCH1 and ROCK1 warrants more research to provide a better understanding of this novel signaling pathway in cell biology. Interestingly, we failed to detect the intracellular form of NOTCH2 after differentiation induction, and ectopic expression of the NOTCH2 intracellular forms had a limited effect, if any, on ROCK1 cleavage and differentiation (Fig. 8A, lane 4), suggesting different regulatory mechanisms for activation and downstream signaling among NOTCH family members.

Functional diversity of caspases in cellular processes other than apoptosis has been described (61). In embryonic mouse keratinocytes, caspase-3 has been shown to be a transcriptional target of Notch1 and to have a role in high commitment to terminal differentiation (62). It is likely that ROCK1 activity is fostered by canonical NOTCH1 signaling through upregulation of caspase-3 gene expression. Because there is also a report showing that caspase-8 functions in epidermal homeostasis and regeneration through regulation of proliferation and inflammatory responses (63), we tested the possible involvement of caspase-8 in ROCK1 cleavage in differentiated keratinocytes. However, caspase-8 inhibition demonstrated little influence on activation of the

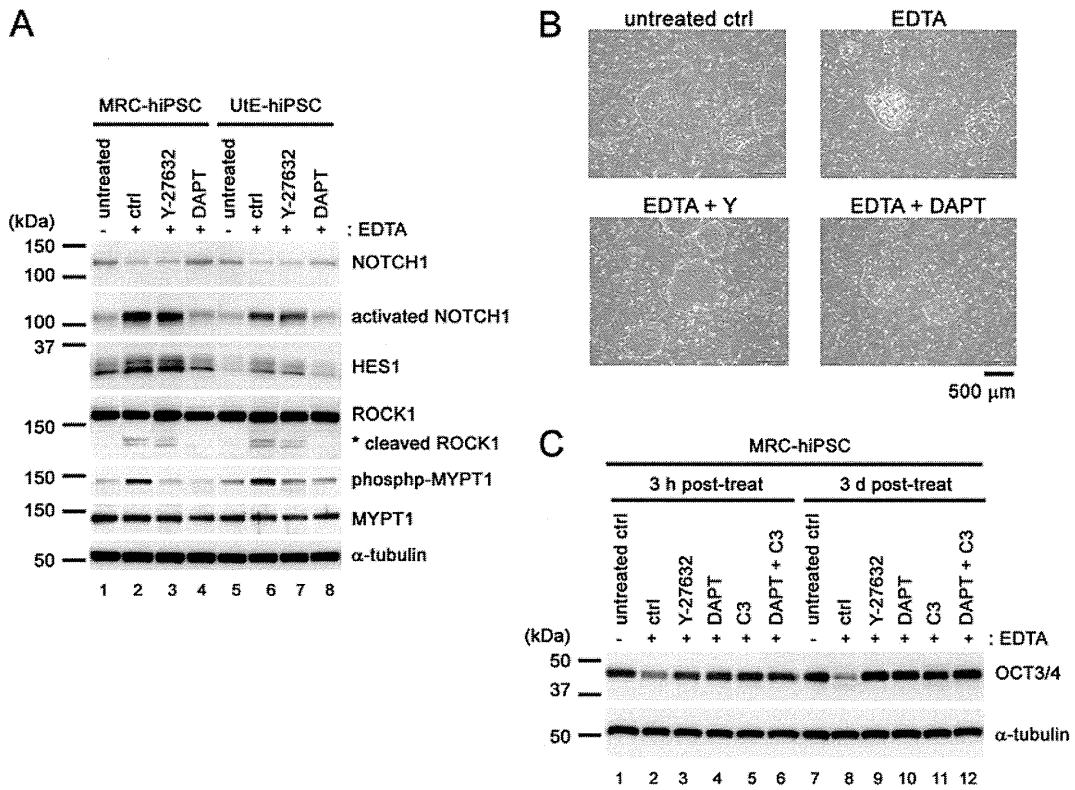

FIG 10 The defect in clonogenic growth upon dissociation in hiPSCs is partly attributable to NOTCH-dependent ROCK activation. (A) Adherent small clumps of MRC-hiPSCs and UtE-hiPSCs were pretreated with 10 μM Y-27632 for 3 h or 10 μM DAPT for 3 h and then either left untreated or treated with 2.5 mM EDTA in Hanks balanced salt solution without Ca^{2+} and Mg^{2+} [HBSS(−)] for 10 min at 37°C. After 3 h of incubation, hiPS cell extracts were prepared and subjected to immunoblotting analysis with the indicated antibodies. (B) Typical MRC-hiPSC colonies were photographed at 3 days after EDTA treatment. (C) Adherent small clumps of MRC-hiPSCs, pretreated where indicated with 10 μM Y-27632 for 3 h, 10 μM DAPT for 3 h, 2.0 μg/ml C3 for 3 h, and 10 μM DAPT and 2.0 μg/ml C3 for 3 h, were then either left untreated or treated with 2.5 mM EDTA. After 3 h and 3 days of cultivation, hiPS cell extracts were prepared and subjected to immunoblotting analysis.

NOTCH1-ROCK1 pathway and keratinocyte differentiation (unpublished observations), suggesting that activation of caspase-3 is unlikely to be mediated by caspase-8 downstream of NOTCH1.

Biological relevance of the NOTCH-ROCK pathway. In stratified epithelia such as the epidermis, cells in the basal layer constitute a proliferating population including stem cells and transit-amplifying cells. In the upper layer, differentiation-committed daughter cells are thought to arise by asymmetric cell division and then move toward the surface to undergo terminal differentiation (64). During the transition from the basal to the suprabasal layer, downregulation of p63, particularly its predominant isoform ΔNp63α, results in detachment from the basement membrane, at least partly via decreased expression of cell adhesion molecules (65). Downregulation of ΔNp63α also leads to upregulation of NOTCH1 gene expression and activity (9, 18). Having established that activated NOTCH1 triggers constitutive activation of ROCK1, we speculate that activated NOTCH1 may drive active movement of daughter cells toward the surface. This idea is supported by experiments showing that active ROCK1 induces formation of thick stress fibers and a cell contraction force which pushes cells upwards (54) and by the fact that Y-27632 inhibits differentiation as well as stratification in organotypic raft culture (37). Indeed, we observed that keratinocytes treated with EDTA exhibited increased cell motility and clambering movement, which were inhibited by Y-27632 (see Movies S1 and S2 in the supplemental material). We previously reported that ΔNp63α represses both p53-dependent and -independent expression of the Notch1 gene to support the proliferative capacity of normal human keratinocytes as well as a subset of cervical cancer cell lines (18). Thus, we propose that the p63-NOTCH1-ROCK1 axis plays an essential role in establishment of stratified epithelia through actomyosin-driven cell movement, though we cannot exclude the possible involvement of the Rho-ROCK pathway in this process. In terms of cancer biology, loss of p63 function is paradoxically associated with metastatic progression (66), implying another face of p63 as a tumor suppressor through the NOTCH1-ROCK1 pathway.

Two isoforms of ROCK, ROCK1 (ROKβ, p160ROCK) and ROCK2 (ROKα), have been identified in mammals; they share 65% overall identity and 92% identity in their kinase domains, and a number of lines of investigation have suggested nonoverlapping functions for these two isoforms (67–69). In this regard, ROCK1, but not ROCK2, has been shown to be constitutively activated by caspase-3 and to play roles in membrane blebbing during apoptosis (54, 55). We observed that knockdown of ROCK1 but not ROCK2 resulted in a reduced propensity for differentiation (Fig. 6B) and that a constitutively active form of ROCK1 induced differentiation (Fig. 8A). In parallel with our data, previous work has shown that the conditional expression of the activated form of ROCK2 also results in induction of differen-

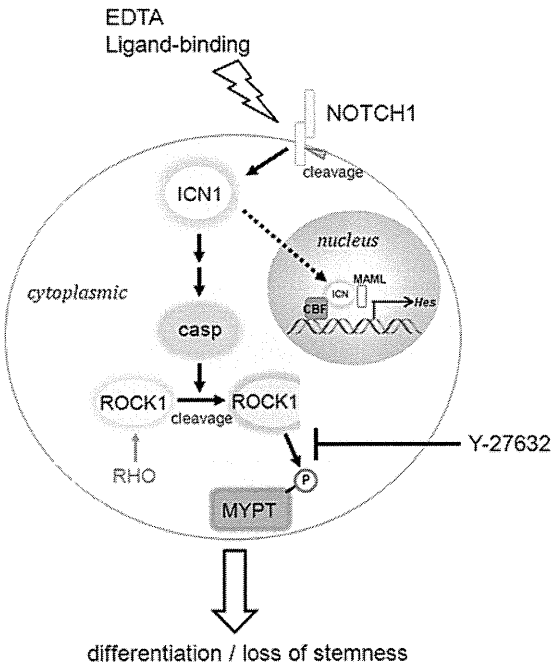

FIG 11 Proposed model for the NOTCH-ROCK pathway and its biological significance. NOTCH1 can be activated by dissociation of cells with EDTA as well as physiological ligand binding. The cytoplasmic form of NOTCH1, independent of its transcriptional activity, triggers caspase-mediated cleavage (activation) of ROCK1 by an as-yet-unknown mechanism. ROCK1 activation drives actomyocin reorganization through MYPT phosphorylation which results in cell differentiation or loss of stemness. The ROCK inhibitor Y-27632 blocks this pathway so as to inhibit differentiation of keratinocytes and maintain stemness of hiPSCs, as shown in this study.

tiation markers in keratinocytes (35). In contrast, it was reported that a ROCK inhibitor accelerated calcium- or suspension-induced keratinocyte differentiation (70). More recently, Notch1 was reported to be a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1,2 and MRCKalpha kinases (56). Furthermore, an oncogenic aspect of ROCK2 has also been reported (52, 71, 72). Apparent anomalies between these studies and our results imply complexity of the signals downstream of ROCK1 and ROCK2. However, our results clearly demonstrate that prompt activation of ROCK1 by noncanonical NOTCH1 signaling promotes keratinocyte differentiation and limits clonogenic cell growth. Part of the discrepancies may be explained by the use of different experimental systems. Our experimental system focused on early stages of keratinocyte differentiation in monolayer culture. However, our results are consistent with a more recent report that ROCK inhibition blocks differentiation as well as stratification in organotypic raft culture (37). It is possible that the canonical NOTCH1 signal and transcriptional repression of ROCK1,2 plays different roles in later stages of differentiation or tumor formation.

In spite of these important functions of ROCK1,2 in keratinocyte differentiation, no abnormalities in the epidermis of either Rock1- or Rock2-null mice have been described (67, 68, 73, 74). This suggests functional redundancy and compensatory mechanisms for these two proteins, though they show apparently quite different functions in culture. Synthetic effects in mice with compound heterozygous and/or homozygous disruptions in the Rock1 and Rock2 genes need to be delineated in detail.

Technical implications of NOTCH-ROCK dysregulation during cell passage. Our data show that keratinocytes of different tissue origins as well as hiPSCs exhibiting epiblast-like cell states have a conserved NOTCH1-ROCK1 pathway and that NOTCH activation is at least partly responsible for impaired clonogenicity upon dissociation with EDTA (Fig. 1C, 9, and 10). ROCK inhibition could robustly restore the proliferation capacity and contribute to the maintenance of stemness through blockade of cellular differentiation and/or apoptosis, unscheduled biological outcomes imposed by the EDTA treatment in cell culture. A recent groundbreaking report provided evidence that the combination of a ROCK inhibitor and fibroblast feeders indefinitely extends the life spans of many different types of human epithelial cells (38). Thus, it is highly likely that dissociation of cells with EDTA activates the NOTCH1-ROCK1 pathway and adversely affects the proliferative capacity of a broad spectrum of normal epithelial cells. Furthermore, most cell lines established through such procedures may represent a selected population resistant to the activation of the NOTCH-ROCK pathway.

In conclusion, our present study unveiled a link between noncanonical NOTCH signaling and ROCK activation and revealed a previously unrecognized function of NOTCH1 as a critical regulator of ROCK1 in dictating the cell fate. Our findings also imply a possible pitfall in cell culture and delineate a molecular rationale for the beneficial effects of the ROCK inhibitor Y-27632 in cultivating various cell types.

ACKNOWLEDGMENTS

We thank H. Sakaguchi, R. Sakamoto, M. Yamazaki, T. Ishiyama, and A. Noguchi for their expert technical assistance and H. Miyoshi for lentiviral constructs.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to T. Yugawa) and by grant 23-B1 from the National Cancer Center Research and Development Fund and a Grant-in-Aid for Cancer Research from the Ministry of Health Labor and Welfare of Japan (to T. Kiyono).

T.Y. conceived the project, designed, performed, and analyzed experiments, and wrote the manuscript. K.N. performed the hiPSC clonogenic assay. N.G. and S.-I.O. provided cDNA clones and performed plasmid construction, respectively. M.F., A.U., and T.N. discussed and gave important advice on some experiments. T.K. participated in the experimental design and performed experiments.

We declare no conflict of interest related to this work.

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Esophagus
DOI 10.1007/s10388-013-0381-9

ORIGINAL ARTICLE

Clathrin heavy chain is a useful immunohistochemical marker for esophageal squamous intraepithelial neoplasia

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Received: 25 February 2013 / Accepted: 19 June 2013

Abstract
Background Recent advances in the endoscopic diagnosis and treatment of esophageal cancer have facilitated the detection and treatment of minute tumors, necessitating the accurate histopathological diagnosis of early esophageal cancer or precancerous lesions. This study evaluated the usefulness of immunohistochemical analysis (IHC) of clathrin heavy chain (CHC) as a marker for early esophageal cancer.
Methods The immunoreactivity of CHC was analyzed in 409 esophageal specimens using a tissue array. Immunoreactivities of CHC, p53, and Ki67 were then compared in 44 endoscopically resected specimens.
Results CHC expression was significantly stronger in the cytoplasm of esophageal squamous cell carcinomas compared with non-tumor specimens in the tissue array. CHC expression in endoscopic specimens was significantly stronger in the cytoplasm of high-grade intraepithelial neoplasias and superficial carcinomas than in benign squamous epithelium and low-grade intraepithelial neoplasias. The sensitivity and specificity of CHC for the diagnosis of esophageal lesions were 75 and 96 %, respectively. These accuracies were comparable with those of p53 (43 and 98 %) and Ki67 (68 and 100 %). In addition, the sensitivity was increased by using a combination of markers as follows: 80 %, CHC + p53; 78 %, CHC + Ki67; 90 %, CHC + p53 + Ki67.
Conclusions CHC detected by IHC may be a useful marker for the pathological diagnosis of esophageal squamous intraepithelial neoplasia.

Keywords Clathrin heavy chain · Endoscopy · Esophageal cancer · Immunohistochemistry · Marker

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Published online: 09 July 2013

Introduction

Early esophageal cancer generally lacks symptoms, and many cases are therefore discovered at an advanced stage when the prognosis is poor [1]. Carcinoembryonic antigen, matrix metalloproteinase-9, squamous cell carcinoma antigen, and p53 are commonly used as diagnostic markers for esophageal cancer [2–5], but their sensitivities and specificities are relatively low, and they are therefore inadequate for identifying early stage esophageal cancer. Esophageal cancer can be detected early by endoscopic screening [6], and lesions identified by endoscopy are then biopsied for histopathological diagnosis. However, determining malignancy by histopathological examination of very small samples, such as biopsy specimens, is difficult, and new pathological diagnostic markers are needed to improve this process.

We recently reported strong expression of clathrin heavy chain (CHC) in the cytoplasm and cell membrane of hepatocellular carcinoma cells, detected by proteome analysis of primary hepatocellular carcinoma (HCC). These results suggest that CHC may be useful as a pathological diagnostic marker for early HCC [7]. CHC plays a major role in cellular endocytosis [8] and is involved in the stability of spindle microtubules in the nucleus [9], which is essential for equipartitioning into sister chromatids and the regulation of p53 transcription activity by binding to p53 [10]. CHC is therefore an interesting protein in relation to carcinogenesis and cancer progression, and may serve as a pathological diagnostic marker for cancers other than HCC. We investigated its immunoreactivity in various esophageal cancer types using tissue arrays. The immunoreactivity of CHC in esophageal cancerous regions was significantly increased, and we therefore evaluated the value of CHC immunoreactivity for the diagnosis of early esophageal cancer. In this study, we subjected tissue arrays and endoscopically resected esophageal tissues to immunohistochemical analysis (IHC) to evaluate CHC immunoreactivity and compared CHC immunoreactivity with those of the current auxiliary diagnostic markers, p53 and Ki67.

Materials and methods

Patients

The subjects were patients who underwent resection at the Department of Frontier Surgery, Chiba University Hospital. Written informed consent was obtained from each patient before surgery. Specimens included mucosal regions resected by endoscopic mucosal resection or endoscopic submucosal dissection in 44 cases. Formalin-fixed paraffin-embedded preparations were used. Preparations were diagnosed by pathologists at our hospital according to the WHO Classification of Esophageal Cancer [11]. Of the 44 cases of mucosal lesions, 12 were low-grade intraepithelial neoplasias (LINs) (including 8 cases in which LIN regions were independently present near malignant regions), 10 high-grade intraepithelial neoplasia (HINs), and 30 superficial carcinomas. Benign squamous epithelia from non-tumorous regions (margins of resected regions) from all 44 cases were used as negative controls.

Tissue array

We used a commercial esophageal tissue array to validate CHC immunoreactivity in esophageal tissues. Tissue arrays (ES2001, ES2084, and ES2082; US Biomax, Inc., Rockville, MD, USA) containing 319 tumor (266 squamous cell carcinoma, 5 adenosquamous carcinoma, 26 adenocarcinoma, and 22 small cell undifferentiated carcinoma) and 90 non-tumor (64 cancer-adjacent normal esophageal tissue, 16 chronic inflammation of esophageal mucosa, and 10 hyperplasia of squamous epithelium) esophageal tissue samples were used for IHC.

Immunohistochemistry

IHC was performed as described previously [7]. Briefly, 4-μm sections from paraffin-embedded tissue were fixed on slide glasses. Anti-CHC mouse monoclonal antibody (BD Biosciences Tokyo, Japan) was diluted 1:200 in blocking buffer (Dako Real™ Antibody Diluent; Dako, Kyoto, Japan). The EnVision + system (Dako) was used to visualize tissue antigens. For double staining of esophageal intraepithelial neoplasia, anti-CHC mouse monoclonal antibody was deactivated by microwave irradiation for 5 min in citric buffer, pH 6.0, after CHC staining, followed by staining with anti-p53 mouse monoclonal antibody (Dako) diluted 1:50 or anti-Ki67 mouse monoclonal antibody (Dako) diluted 1:100 in blocking buffer (Dako Real™ Antibody Diluent). The EnVision G|2 System/AP (Rabbit/Mouse (Permanent Red); Dako) was used to visualize tissue antigens. CHC-positive regions were stained brown in the cytoplasm, and p53- and Ki67-positive regions were stained red with Permanent Red in the nucleus. The staining intensity was classified as negative or positive: For CHC, intense staining more than 30 % was judged as positive (Fig. 1). For p53, intense staining more than 30 % was judged as positive. For Ki67, the mucosa was divided into the upper and lower (basal) layers and staining was judged in each layer. Staining more than 30 % was judged as positive. IHC of the samples was evaluated by our two pathologists (T.T., Y.N.).

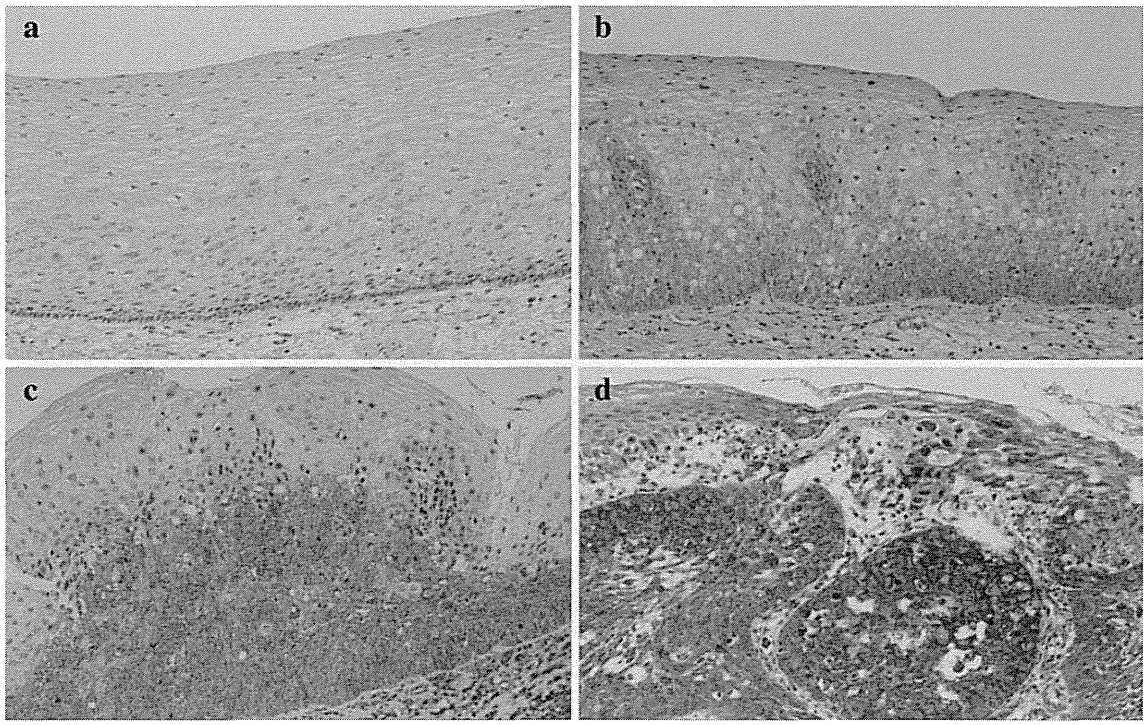

Fig. 1 The staining intensity of clathrin heavy chain (CHC) **a** negative (benign squamous epithelia), **b** negative (low grade dysplasia), **c** positive (superficial carcinoma), and **d** positive (advanced carcinoma)

Table 1 Immunoreactivity of CHC in tissue array

CHC expression level	Non-tumor			Tumor			
	Normal	Chronic inflammation	Hyperplasia	Squamous cell carcinoma	Adenosquamous carcinoma	Adeno-carcinoma	Small cell undifferentiated carcinoma
Negative	63	15	10	119	3	7	15
Positive	1	1	0	147	2	19	7

Results

Immunoreactivity of CHC in the tissue array

CHC expression was positive in many esophagus carcinoma tissue samples (55 %). In contrast, most non-tumor tissues (98 %) were negative (Table 1).

Immunoreactivities of CHC, p53, and Ki67 in endoscopic specimens

IHC double staining of CHC-p53 and CHC-Ki67 is shown in Fig. 2. CHC was positive in 75 % of HINs or superficial carcinomas, and p53 was positive in 43 % of HINs or superficial carcinomas. Ki67 was positive in the lower layer of the esophageal epithelium in 66 % cases of benign squamous epithelium or LIN. However, no expression was noted in the upper layer of benign squamous epithelium or LIN. Positive staining was noted in the lower (93 %) and upper layers (68 %) in many cases of HIN or superficial carcinoma (Table 2).

We set the expression conditions of the markers to differentiate between malignant (HIN and superficial carcinoma) and benign lesions (benign squamous epithelium and LIN). Positivity was judged to be the criterion for differentiating between malignant and benign lesions in terms of CHC, with a sensitivity and specificity of 75 and 96 %, respectively. Similarly, positivity was the optimal p53 criterion, with a sensitivity and specificity of 43 and 98 %, respectively. Positivity in the upper layer was the optimal criterion for Ki67 protein staining, with a sensitivity and specificity of 68 and 100 %, respectively. The sensitivity could be increased by using a combination of these markers (Table 3). There were significant differences in the immunoreactivities of all proteins between squamous epithelium/LIN and HIN/superficial carcinoma ($p < 0.001$).

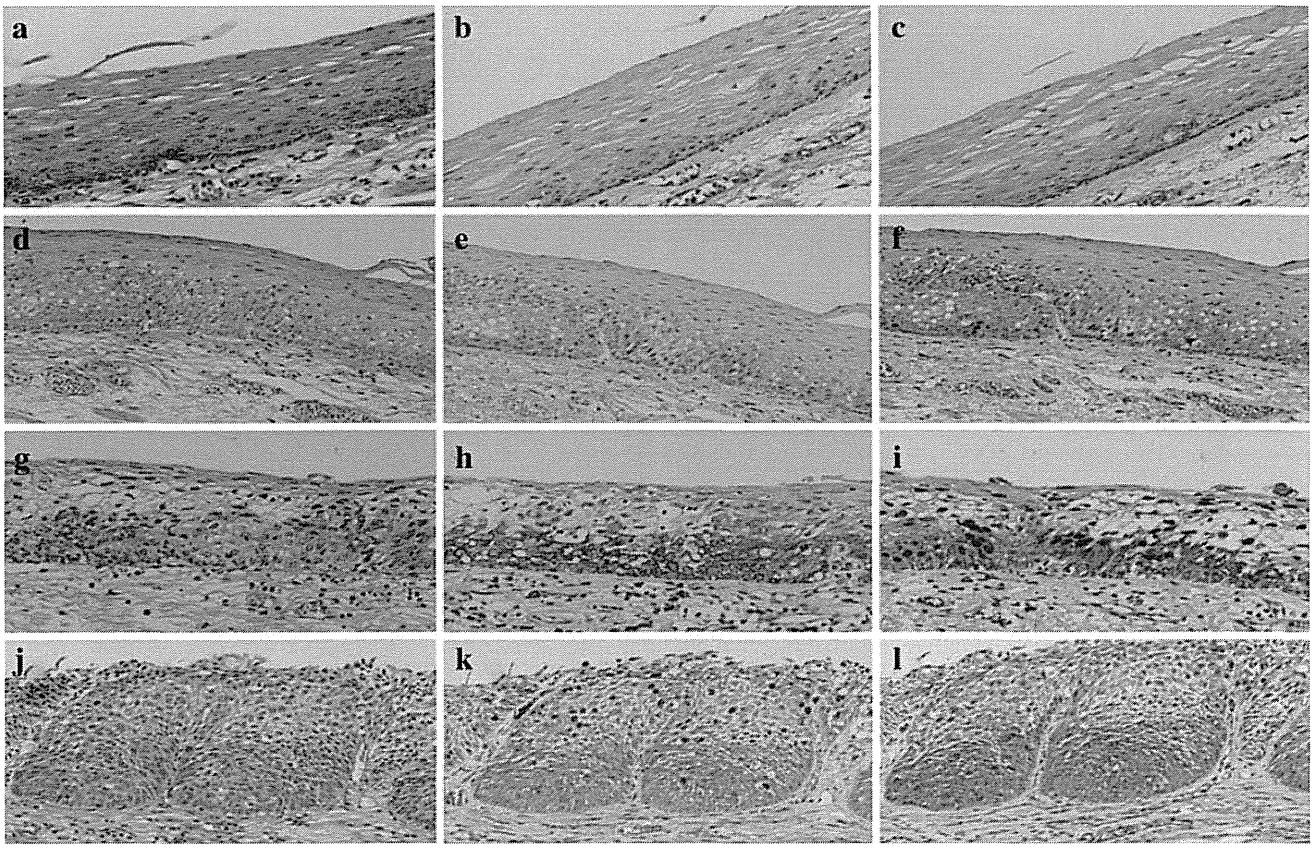

Fig. 2 Immunohistochemical double staining of esophageal epithelium CHC was stained brown in the cytoplasm, and p53 or Ki67 was stained red in the nucleus. **a–c** Benign squamous epithelial region, **d–e** low-grade intraepithelial neoplasia, **g–i** high-grade intraepithelial neoplasia, and **j–l** superficial carcinoma (carcinoma invading the lamina propria). **a, d, g, j** Conventional hematoxylin-eosin staining, **b, e, h, k** immunohistochemical double staining with CHC and p53, and **c, f, i, l** immunohistochemical double staining with CHC and Ki67

Table 2 Immunoreactivity of proteins in mucosal lesions

Marker	Immunoreactivity	Benign lesions		Malignant lesions	
		Benign squamous epithelium	LIN*1	HIN*2	Superficial carcinoma
CHC	Negative	42	12	3	7
	Positive	2	0	7	23
P53	Negative	43	12	7	16
	Positive	1	0	3	14
Ki67 (in lower layer)	Negative	16	3	1	2
	Positive	28	9	9	28
Ki67 (in upper layer)	Negative	44	12	5	8
	Positive	0	0	5	22

* 1, low-grade intraepithelial neoplasia; * 2, high-grade intraepithelial neoplasia

Discussion

The risk of developing invasive cancer in LIN (without basal layer type squamous cell carcinoma in situ) is low, while HIN, in contrast, is likely to progress to invasive cancer and thus requires early treatment [12–16]. Differentiating between LIN and HIN is therefore very important. Unfortunately, however, it is difficult to make an accurate histopathological diagnosis based on small tissue samples. p53 and Ki67 are currently used as immunohistochemical esophageal markers, but there have been very few reports on immunohistochemical markers of esophageal intraepithelial tumors [17], and there is no clear information regarding the differentiation of benign squamous

Table 3 Diagnostic accuracy of immunoreactivity employing each protein alone and in combination

Marker	Sensitivity (%)	Specificity (%)
CHC	75	95
p53	43	98
Ki67	68	100
Combination		
CHC and p53	80	95
CHC and Ki67	78	95
p53 and Ki67	78	98
CHC and p53 and Ki67	90	95

* Decision criterion for differentiating between high-grade intraepithelial neoplasia/superficial carcinoma and benign squamous epithelium/low-grade intraepithelial neoplasia/LIN

epithelium/LIN from malignant lesions (HIN/superficial carcinoma).

We investigated the usefulness of CHC IHC for the histological diagnosis of esophageal cancer in 409 cases, using commercial esophageal tissue arrays. CHC was positive in only 2 % of benign squamous epithelia, but in 55 % of malignant cases. Apparent differences in CHC expression were noted between mucosal preparations of benign squamous epithelium/LIN and HIN/superficial carcinoma. The sensitivity and specificity of CHC for the diagnosis of esophageal lesions were 75 and 96 %, respectively, indicating the usefulness of CHC as an immunohistochemical marker of esophageal epithelial lesions.

IHC double staining of CHC/p53 and CHC/Ki67 was used to compare CHC with p53 and Ki67, which are currently used as auxiliary diagnostic markers of esophageal cancer. p53 is a tumor suppressor protein, but wild-type p53 is rapidly degraded and is normally present in the nucleus at a very low level. In comparison, mutant p53 protein has markedly delayed intracellular degradation and accumulates in the nucleus, allowing its detection by IHC [18–20]. Residual p53 protein is observed in early esophageal squamous cell carcinomas and precancerous lesions. A high incidence of mutant p53 has recently been demonstrated in LIN and HIN tissues by laser capture microdissection [21]. In the current study, p53 staining was slightly positive in the lower (basal) layer in 17 % of LIN cases (data not shown), but no regions showed positivity. In contrast, the lower layer demonstrated positivity in 30 % of HIN cases, suggesting that not only the presence/absence of mutant p53, but also the level of residual p53 protein in the nucleus may be associated with carcinogenesis and cancer progression.

Ki67 protein is expressed in the G1, S, G2, and M phases, but not in the G0 phase during cell division, showing that an increase in Ki67 protein indicates active cell proliferation, and high Ki67 expression levels have been reported in tumor cells [22, 23]. Ki67 was positive in the lower layer of the esophageal epithelium in many cases, reflecting active cell division in the lower layer, while Ki67 was negative in the upper layer of tissue samples in benign squamous epithelium and LIN, but positive or in many cases of HIN, suggesting active cell division in the upper layer of the esophagus in HIN.

We investigated the optimal condition for each marker in terms of differentiating between benign (benign squamous epithelium/LIN) and malignant lesions (HIN/superficial carcinoma). Positivity for p53 and positivity in the upper layer of the mucosa for Ki67 were considered to be optimal conditions, respectively. The sensitivity and specificity of CHC alone were 75 and 96 %, respectively, which were comparable with or superior to those of p53 (43 and 98 %) and Ki67 (68 and 100 %). In addition, the sensitivity could be increased using a combination of these markers, to 80 % for CHC + p53, 78 % for CHC + Ki67, and 90 % for CHC + p53 + Ki67.

These results suggest that CHC IHC could be a useful histological auxiliary diagnostic technique for differentiating between benign squamous epithelium/LIN and HIN/superficial carcinoma in the histopathological diagnosis of esophageal intraepithelial tumors. CHC IHC might contribute to the accurate pathological diagnosis of esophageal cancers that are difficult to diagnose by conventional hematoxylin-eosin staining. The mechanisms by which the expression levels of CHC are enhanced in esophageal squamous cell carcinoma remain to be investigated. Clarification of these mechanisms may lead to further understanding of the tumor biology of esophageal cancers.

Acknowledgments This study received financial support from a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (no. 22590518 and 25460675).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard statement The authors work conformed to the guidelines set forth in the Helsinki Declaration of 1975, as revised in 2000.

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Gastric Cancer (2014) 17:26–33
DOI 10.1007/s10120-013-0259-5

REVIEW ARTICLE

Clinical significance of serum tumor markers for gastric cancer: a systematic review of literature by the Task Force of the Japanese Gastric Cancer Association

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Received: 25 December 2012 / Accepted: 24 March 2013 / Published online: 10 April 2013

Abstract The aim of this review was to evaluate the clinical significance of serum tumor markers, particularly CEA, CA19-9, and CA72-4, in patients with gastric cancer. A systematic literature search was performed using PubMed/MEDLINE with the keywords "gastric cancer" and "tumor marker," to select 4,925 relevant reports published before the end of November 2012. A total of 187 publications contained data for CEA and CA19-9, and 19 publications contained data related to all three tumor markers. The positive rates were 21.1 % for CEA, 27.8 % for CA19-9, and 30.0 % for CA72-4. These three markers were significantly associated with tumor stage and patient survival. Serum markers are not useful for early cancer, but they are useful for detecting recurrence and distant metastasis, predicting patient survival, and monitoring after surgery. Tumor marker monitoring may be useful for patients after surgery because the positive conversion of tumor markers usually occurs 2–3 months before imaging abnormalities. Among other tumor markers, alpha-fetoprotein (AFP) is useful for detecting and predicting liver metastases. Moreover, CA125 and sialyl Tn antigens (STN) are useful for detecting peritoneal metastases. Although no prospective trial has yet been completed to evaluate the clinical significance of these serum markers, this literature survey suggests that combinations of CEA, CA19-9, and CA72-4 are the most effective ways for staging before surgery or chemotherapy. In particular, monitoring tumor markers that were elevated before surgery or chemotherapy could be useful for detection of recurrence or evaluation of the response.

Keywords CA19-9 · CA72-4 · CEA · Gastric cancer · Serum tumor marker · Systematic review

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Introduction

The Japanese Public Health Insurance System covers the costs of monitoring patients with gastric cancer using serum tumor markers. Nine types of serum markers are officially certified for use in disease monitoring: carcinoembryonic antigen (CEA) in the sialyl Lewis A group; CA19-9 and CA50 in the sialyl Lewis Tn group; STN and CA72-4 in the mucin antigen group; and CA125, alpha-fetoprotein (AFP), IAP, and TPA. Many studies have demonstrated the clinical significance of each marker; however, appropriate indications for serum tumor marker monitoring remain unclear. The serum levels of CEA,

CA19-9, and CA72-4 may be elevated in patients with gastric cancer at various stages [1]. AFP [2], CA125 [3], and STN [4] can be used to detect liver metastases and/or peritoneal metastases. However, low rates of sensitivity and specificity prevent the use of any of these serum markers in early diagnosis. The National Comprehensive Cancer Network guidelines (http://www.nccn.org) do not recommend serum marker testing for preoperative evaluation and staging of gastric cancer.

In this context, the Task Force of the Japanese Gastric Cancer Association for Research Promotion (directed by Dr. Motoki Ninomiya) planned to reevaluate the clinical impact of serum tumor markers in a systematic review of previous publications, focusing mainly on CEA, CA19-9, and CA72-4. The clinical significance of the three other serum markers, AFP, CA125, and STN, was also addressed. Prospective clinical studies can be planned based on the results of this systematic review to elucidate the clinical utility of serum tumor markers.

Manuscript selection

A computer-aided search of the PubMed website (http://www.ncbi.nlm.nih.gov/sites/entrez) was conducted to retrieve relevant articles on serum tumor markers used for gastric cancer. The keywords "gastric cancer" and "tumor marker" and the serum markers CEA, CA19-9, CA72-4, AFP, CA125, STN, TPA, and IAP were used to search for relevant articles published before the end of November 2012 (Table 1). Studies investigating the clinicopathological impact of preoperative serum tumor markers used for assessing patients with gastric cancer were selected. Furthermore, case reports, review article, non-English articles, articles that included less than 30 patients, and articles that addressed cancers other than gastric cancer were excluded. Four researchers (H.S., T.N., M.O., and Y.T.) reviewed all the articles, and after applying the inclusion and exclusion criteria arrived at a consensus about articles to be selected at a working meeting. A total of 657 articles were selected from the PubMed database using the keyword "CEA." A total of 46 articles were selected as references for the present review article to evaluate the positive rates for CEA (n = 8,104), CA19-9 (n = 5,300), and CA72-4 (n = 2,774) [4–49]. In this review, the number of positive patients reported as positive, based on the definition in each original paper, was used to calculate the combined positive rates of patients with early/advanced gastric cancer. The positive rates for each marker at each stage were calculated. Among these 46 articles, 19 articles [1, 9, 12, 15, 19, 20, 22, 26, 27, 31, 32, 34–37, 39, 42, 43, 50] analyzed all three markers, CEA, CA19-9, and CA72-4, which included 2,774 patients (Table 2). Four discussion points were evaluated for CEA, CA19-9, and CA72-4 from selected articles as follows: (1) positive rates, (2) clinicopathological significance, (3) prognostic impact, and (4) clinical impact during follow-up after surgery and/or during chemotherapy. Finally, we selected 10 other publications that focused only on AFP [51–54], CA125 [3, 55–57], and STN [58, 59] in patients with gastric cancer to analyze the clinical significance of these three serum markers.

Table 1 Key words with "gastric cancer and tumor marker" and number of publications from PUBMED search

Key words	Number of publications
CEA	657
CA19-9	281
CEA + CA19-9	187
AFP	179
CA125	44
CEA + AFP	42
CEA + CA125	28
CA72-4	26
STN	26
CEA + CA72-4	25
CEA + CA19-9 + CA72-4	24
IAP	21
CA50	7

Positive rates for each serum marker according to the TNM stages

In the initial 46 articles, the overall positive rates for each marker were as follows: 24.0 % (1,945/8,104) for CEA, 27.0 % (1,431/5,300) for CA19-9, and 29.9 % (829/2,774) for CA72-4. The positive rates for CEA during each stage were as follows: stage I = 13.7 %, stage II = 23.0 %, stage III = 25.6 %, and stage IV = 39.5 %. The positive rates for CA19-9 during each stage were as follows: stage I = 9.0 %, stage II = 19.9 %, stage III = 32.2 %, and stage IV = 44.7 %. The positive rates for CA72-4 during each stage were as follows: stage I = 12.0 %, stage II = 15.6 %, stage III = 36.7 %, and stage IV = 49.6 % (Fig. 1).

The 19 manuscripts that evaluated all three markers in the same group of patients (n = 2,774) showed a similar trend as shown in Fig. 1 (Table 2). The positive rate for CA72-4 was the highest among the three markers (Fig. 2). Among these 19 articles, 12 articles indicated that the positive rate for CA72-4 was the highest among the three serum markers (Table 2). The combination with the highest positive rate was CA19-9 and CA72-4 [15, 50]. Because the average frequency of these three markers was approximately 10 % at stage I (CEA = 13.7 %, CA19-

Table 2 A total of 19 publications analyzed all three serum markers CEA, CA19-9, and CA72-4 in patients with gastric cancer

Reference	Author	Journal	Year	Number of patients	CEA (%)	CA19-9 (%)	CA72-4 (%)
9	Guadagni F	Cancer Res	1992	94	20	32	43
12	Guadagni F	Anticancer Res	1993	161	42	34	22
15	Filella X	Acta Oncol	1994	79	33	46	47
19	Fernandez-Fernandez L	Int Surg	1996	167	21	26	60
20	Spila A	Anticancer Res	1996	242	22	33	41
22	Pectasides D	Am J Clin Oncol	1997	62	49	65	70
26	Tocchi A	J Cancer Res Clin Oncol	1998	59	58	39	19
28	Marrelli D	Oncology	1999	254	21	35	28
31	Marrelli D	J Surg Oncol	2001	167	16	34	20
32	Marrelli D	Am J Surg	2001	133	16	35	20
34	Gaspar MJ	Tumour Biol	2001	82	16	33	34
35	Mattar R	Rev Hosp Clin Fac Med Sao Paulo	2002	44	25	25	48
36	Lai IR	Hepatogastroenterology	2002	195	32	16	16
37	Aloe S	Int J Biol Markers	2003	166	23	25	37
39	Louhimo J	Int J Cancer	2004	146	18	31	34
42	Goral V	Hepatogastroenterology	2007	47	30.5	30	46.8
43	Ucar E	Adv Ther	2008	95	24.2	41	32.6
1	Kim DH	J Surg Oncol.	2011	312	1	1	5
1	Kim DH	J Surg Oncol.	2011	167	5	13	15
50	Emoto S	Gastric Cancer	2012	102	19	37	44.9

Reference [1] presented data of "early" and "advanced" tumors separately

Positive rates are shown as %

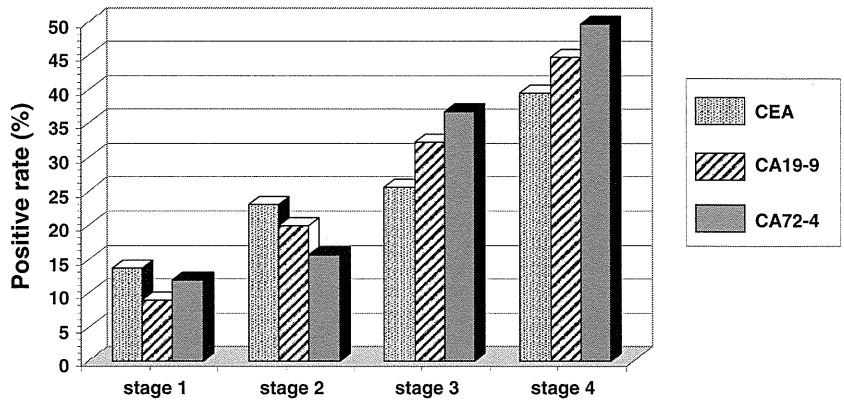

Fig. 1 Positive rate of serum tumor markers in gastric cancer according to stage

9 = 9.0 %, and CA72-4 = 12.0 %), they may not be useful for early cancer screening. Although the positive rates for CEA, CA19-9, and CA72-4 were similar in detecting major tumors, CA72-4 had the highest positive rate in patients with nodal involvement or serosal invasion. Therefore, CA72-4 was the most useful marker for detecting advanced gastric cancer [34, 39, 42, 43].

A recent meta-analysis of Chinese studies also showed that CA72-4 was the best of these three serum markers [60]. The accumulated accuracy rate of CA72-4 was 77 %, which was better than others. CA72-4 was the most highly correlated serum tumor biomarker for gastric cancer in the Chinese population. A combination of CA72-4 + CEA + CA19-9 considerably improved the positive rate without impairing the specificity.

Association of elevated serum markers with clinicopathological factors

CEA

The overall positive rates for CEA were 16–68 %. CEA was strongly associated with the T factor [29, 30, 33, 37],

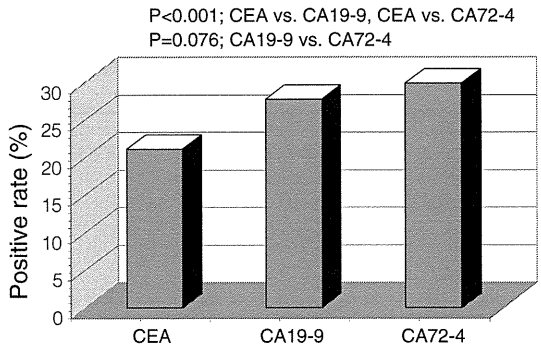

Fig. 2 Positive rate of each serum tumor marker in gastric cancer: In 19 articles, a total of 2,774 patients showed positive rates of all three serum markers

N factor [18, 24, 27, 29, 30], M factor [5, 6, 11, 24, 27, 33, 43], and stage [18, 27, 29, 39, 40, 45, 48]. Ikeda et al. [11] analyzed 68 patients with stage IV gastric cancer using multivariate analysis and concluded that an elevated CEA level was an independent risk factor for predicting liver metastases. A few reports have shown a significant association between elevated CEA and peritoneal metastases [18, 34]. Although several reports showed that elevated CEA was significantly associated with differentiated tumor types [7, 10, 48, 61], a few reports indicated an association with poorly differentiated types of tumors [29]. Maehara et al. analyzed the CEA levels in 221 patients with well-differentiated gastric cancer. The CEA-positive patients had larger tumors, greater serosal invasion, more frequent lymphatic and vascular involvement, less expansive tumor growth, and higher rates of lymph node and hepatic metastases than CEA-negative patients [17].

CA19-9

The overall positive rates for CA19-9 were reported as 14–68 %. Elevated CA19-9 was associated with tumor depth [30, 37], nodal involvement [18, 34, 40, 43, 47, 48], peritoneal metastases [18, 34, 43], and stage [18, 30, 34, 43, 47]. Of these various clinicopathological factors, CA19-9 was frequently reported to be associated with nodal involvement. The positive predictive value for nodal involvement was reported to be 78–96 % [18, 43, 47]. The positive predictive value for peritoneal metastases was reported to be 27 % [18] or 24 % [17]. Kodera et al. [18] reported that elevated CA19-9 levels were strongly associated with liver metastases.

CA72-4

The overall positive rates for CA72-4 were reported to be 16–70 %, which were generally higher than CEA and CA19-9 [1, 12, 37, 39, 42, 50]. Elevated CA72-4 was associated with tumor depth [28, 37, 43], nodal involvement [10, 12, 20, 24, 28, 34, 37, 42, 43], peritoneal metastases [10, 24, 34, 43], distant metastases [34, 43], and stage [9, 20, 28, 34, 35, 39, 42, 43]. Ucar et al. [43] reported that 9 of 11 patients (82 %) with liver metastases were positive for CA72-4. Because the positive rate for CA72-4 in patients with poorly differentiated adenocarcinoma was significantly higher than that for CEA (36 vs. 8 %), the overall positive rate for CA72-4 was higher than that for CEA [25]. In patients with Bormann type 2, 3, and 4, the positive rates for CA72-4 were higher than that for CEA. In particular, the positive rate for CA72-4 was significantly higher than that for CEA in patients with Bormann type 4 (67 vs. 11 %) [10]. The positive rate for CA72-4 was higher than that for CEA in stage III or IV patients. In particular, the positive rate for CA72-4 was significantly higher than that for CEA in patients with peritoneal metastases (69 vs. 23 %) [10].

Association of elevated serum markers with recurrence and patient survival

Because elevated serum markers were generally associated with tumor progression, most previous reports concluded that preoperative elevated serum markers were significantly associated with poor long-term patient survival. The prognostic value of preoperative CEA was confirmed by univariate analysis [18, 27, 28, 30, 34, 43, 44] and multivariate analysis using TNM factors [13, 14, 23, 25, 26, 62]. The prognostic value of preoperative CA19-9 was also confirmed by univariate analysis [25, 27, 28, 30, 31, 34, 39, 40, 43] and multivariate analysis using TNM factors [18, 26, 29, 44, 63]. The prognostic value of preoperative CA72-4 was also confirmed by univariate analysis [23, 24] and multivariate analysis using TNM factors [33, 39, 43, 63]. Although none of the three markers was associated with peritoneal recurrences, preoperative positivity for CEA, CA19-9, or CA72-4 was an independent risk factor for hematogenous recurrences of gastric carcinoma, and this point should be considered when selecting adjuvant chemotherapy after surgery for gastric cancer [1, 24, 32, 37]. Among these three markers, preoperative elevated CA72-4 was an independent risk factor for reduced patient survival in a multivariate analysis when co-analyzed with CEA and CA19-9 [63].

Takahashi et al. [38] reported that the CEA levels and/or CA19-9 levels increased for the first time at recurrence (54.7 and 40.0 %, respectively). Sensitivities for CEA and CA19-9, and combinations of the two markers, for indicating recurrence were 65.8, 55.0, and 85.0 % [38]. More than 90 % of patients with elevated preoperative levels of CEA had increased CEA levels again at the time of

recurrence. Similarly, the CA19-9 level increased again at recurrence in more than 90 % of patients with high preoperative levels of this marker [38]. Kim et al. confirmed these findings based on follow-up data from 1,117 patients. They concluded that the postoperative elevation of CEA and/or CA72-4 were both independent risk factors for recurrence [1]. Liu et al. [64] also reported that CA72-4 was the highest in sensitivity (35 %) and that the combined triple markers had 62 % sensitivity in the diagnosis of recurrence. They also reported false-positive rates of CEA, CA19-9, CA72-4, and the triple markers were 5.6, 7.0, 9.9, and 18.3 %, respectively [64]. Choi et al. [65] reported that the majority (90 %) of cases with recurrence to the liver had an elevated CEA, whereas an elevated CA 19-9 postoperatively was more predictive of a peritoneal recurrence (78.9 %). CA19-9 may be particularly useful as a marker of peritoneal recurrence, whereas CEA could be a useful marker for recurrence in the liver [65].

Therefore, the patients should have a set of markers evaluated once preoperatively. Measurement during the postoperative follow-up would then be particularly important for those who had elevated preoperative values, although one cannot deny the relevance of measuring tumor markers among patients who did not have an elevated preoperative value.

Doubling time and lead time of elevated serum markers

The reported doubling time estimate, based on the serum level of CEA, agreed with the actual tumor doubling time in 112 previously untreated patients with recurrent gastric cancers [66]. The CEA doubling time ranged from 12 to 105 days, with a mean of 37.5 days. The CEA doubling time was significantly shorter in patients with papillary adenocarcinoma compared with those with well- or moderately differentiated tubular adenocarcinoma. The doubling time was also significantly shorter in patients with liver metastasis than those with lymph node metastasis or peritoneal dissemination. There was also a significant correlation between the CEA doubling time and postoperative survival time of patients who received no chemotherapy.

The serum markers were frequently elevated several months before imaging abnormalities. Thus, the lead time before imaging abnormalities was reduced gradually because of improvements in imaging technology. In 1982, Tamada et al. [5] reported that the lead time for CEA was 8.3 months. Because of improvements in imaging modalities, recent studies (after 2000) reported a shorter mean lead time than that reported earlier (3–5 months for CEA and 2–5 months for CA19-9) [31, 38]. The preoperative seropositive group was more likely to have a longer lead time than the preoperative seronegative group. The lead times for recurrences in the liver, peritoneum, and lymph nodes were 1.2, 3.4, and 3.7 months, respectively, for CEA and 2.1, 1.0, and 3.6 months, respectively, for CA19-9. The lead time for CEA for liver recurrence was significantly shorter than those for peritoneum and lymph node metastases [38]. It was concluded that because the lead time depends on the imaging modalities and follow-up interval, a large-scale prospective study is required to clarify the best strategy for follow-up to improve overall patient survival.

Clinical significance of serum marker monitoring during chemotherapy

Yamao et al. [66] monitored changing patterns in CEA, CA19-9, and CA125 levels during systemic chemotherapy to determine the relationship between changes in the serum tumor marker levels and the response assessment in imaging studies throughout the treatment course. The sensitivity and negative predictive value of falling tumor marker levels after chemotherapy for a partial response in imaging was 100 %. On categorizing the patients as responders or nonresponders, a significant correlation was observed between the assessment of response by tumor markers and by imaging studies. The survival time of responders assessed by tumor markers was significantly longer than that of nonresponders [66]. Catalano et al. analyzed the CEA levels in 175 patients with advanced gastric cancer who received second-line chemotherapy. Univariate and multivariate analyses showed that elevated CEA levels >50 ng/ml were significantly associated with poor overall survival. This analysis suggests that readily available clinical factors may help to select patients with advanced gastric cancer who may benefit from second-line chemotherapy [67].

Regarding elevated serum marker levels immediately after chemotherapy, Kim et al. [68] reported a transient increase in the CEA or CA19-9 levels despite the clinical benefits of chemotherapy in patients with metastatic or recurrent gastric cancer. CEA and CA 19-9 surges were defined as >20 % increases in these tumor marker levels from the baseline, followed by >20 % drop in subsequent levels compared with the baseline. Of 51 patients who were evaluated for CEA surges, nine (18 %) patients had CEA surges. The median time to the CEA peak and the duration of the CEA surge were 2.8 and 9.1 weeks, respectively. Of 40 patients who were evaluated for CA19-9 surges, 7 (18 %) had CA19-9 surges. The median time to the peak and the duration of the CA19-9 surge were 2.3 and 7.1 weeks, respectively. All patients with these surge phenomena received clinical benefits from chemotherapy. Although increases in serum tumor markers after chemotherapy were general indicators of tumor progression, an initial rise in the CEA or CA19-9 levels after the initiation

Table 3 Clinical significance of serum tumor markers in gastric cancer

	T	N	M	P	Histology	Prognosis	Recurrence pattern
CEA	Yes	Yes	Yes	No	Yes	Yes	Distant
CA19-9	Yes	Yes	Yes	Yes	No	Yes	Distant
CA72-4	Yes	Yes	Yes	Yes	No	Yes	Distant and/or peritoneal
AFP	NA	NA	Yes	NA	Yes	Yes	Liver
CA125	NA	NA	NA	Yes	No	Yes	Peritoneal
STN	NA	NA	Yes	Yes	No	Yes	Peritoneal

NA not enough evidence to evaluate clinical significance was available

of chemotherapy should not be an indicator of progressive disease in some cases [68].

Other useful serum markers for gastric cancer: alpha-fetoprotein (AFP), CA125, and sialyl Tn antigens (STN)

AFP-producing gastric cancers behave aggressively and have a high potential for metastasis to the liver [2, 52–54]. There was poorer differentiation, a higher incidence of lymph node metastasis, and more marked lymphatic and vascular invasion in the AFP-positive group than in the AFP-negative group [54].

The diagnostic ability of the serum CA125 was more reliable than other imaging modalities including computed tomography, ultrasonography, and the other serum tumor markers for peritoneal metastasis from gastric carcinoma [3]. The predictive values of the serum CA125 levels at a cutoff value of 35 U/ml resulted in a sensitivity of 39.4 %, a specificity of 95.7 %, and a diagnostic accuracy of 90.8 %. Hwang et al. [57] analyzed the utility of diagnostic imaging and CA125 levels in the sera of 768 patients with gastric cancer. The serum CA125 levels had high sensitivity (38.6 %), specificity (98.4 %), and diagnostic accuracy (91.5 %). Emoto et al. [50] also showed the sensitivities of CA125 for peritoneal metastasis at the initial diagnosis was 46 %. The CA125 level was significantly correlated with the degree of peritoneal dissemination and patient survival [50, 55, 57].

Takahashi et al. [4, 58] evaluated the clinical significance of the serum STN level as a tumor marker in 350 patients with gastric cancer. Histologically, the tumors in the high STN group were deeply penetrating and the rates of lymphatic involvement, vascular involvement, and lymph node and hepatic metastases were higher. The 5-year survival rate for patients in the high STN group was significantly less than that of patients in the low STN group (44.8 vs. 75.1 %, $P < 0.05$). Nakagoe et al. [59] confirmed similar conclusions that high serum STN was an independent factor that predicted liver metastasis and a worse outcome in gastric cancer patients.

Because these three markers could classify advanced gastric cancer into a specific category, measurement during the postoperative follow-up would be particularly important for those who had elevated preoperative values.

In conclusion, this systematic review evaluated 657 publications related to serum tumor markers in patients with gastric cancer (Table 3). Although no prospective trial has yet been completed to evaluate the clinical significance of these serum markers, this literature survey suggests that combinations of CEA, CA19-9, and CA72-4 are the most effective ways for staging before surgery and chemotherapy. Monitoring those positive markers after treatment should be important. AFP is useful for detecting and predicting liver metastases. CA125 and STN are useful for detecting peritoneal metastases. Any of these serum markers may be a risk factor for poor patient survival. Final conclusions about the clinical utility of these serum markers for patients with gastric cancer during treatment should be clarified in a phase III prospective randomized trial for certain anticancer agents or radical surgery.

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