677 exhibit reduced serum folate levels, while carriers of the A1298C variant allele have been supposed to have increased folate levels [4,5,10]. Additionally, the intake of some nutrients involved in the folate metabolic pathway (e.g., vitamins B_6 and B_{12} and methionine), as well as alcohol (a folate antagonist), may modify the effect of *MTHFR* polymorphisms on folate serum levels, possibly affecting cancer risk [11].

Fruit and vegetables have been hypothesized to protect against areo-digestive cancers [12], with the chemopreventive effect possibly due to their high content of some vitamins and folate [13,14]. However, the results are not yet conclusive due to possible confounding by effect by dietary or lifestyle factors. Studying the association between sequence variants of folate related genes on cancer risk has the advantage of being less prone to the confounding effect of environmental covariates [15]. As such, several studies have investigated the association between the two common *MTHFR* polymorphisms and the risk of lung and head and neck cancer [6,7,16–33], with many of them relatively small in size. A very recent meta-analysis reported the absence of association between *MTHFR* C677T and A1298C and lung cancer [34], however authors did not include all the published reports available on *MTHFR* C677T at the time it was published, and were unable to stratify the results according to folate status and alcohol consumption.

The aim of the present meta-analyses is to assess the overall effect of the *MTHFR* C677T and A1298C polymorphisms on head and cancer, as well as the potential effect modification of C677T variants by folate intake and alcohol consumption. Additionally, authors aim to integrate the previous meta-analysis on C677T and lung cancer [34] with all published papers, and test for interaction with folate and alcohol where possible. Lastly, results of the meta-analysis of *MTHFR* A1298C and lung cancer are presented for completeness.

2. Materials and methods

2.1. Inclusion criteria

Identification of the studies was carried out through a search of Medline and Embase up to 31st December 2007 using the following terms: (methylenetetrahydrofolate reductase or MTHFR) and (lung or (neck and head) or "head and neck neoplasms"[MeSH]) and (cancer or carcinoma), without any restriction on language. A cited reference search of the retrieved articles was carried out, and publications were also identified by reviewing the bibliographies of the retrieved articles. Eligible studies were community-based that reported the frequency of the *MTHFR C677T* or *A1298C* polymorphisms as number of cancer cases and controls according to the three variant genotypes of either polymorphisms. For those papers reporting only the allele frequencies, the corresponding authors were contacted by email in order to obtain the absolute number of homozygous and heterozygous individuals in case and control groups. Studies whose allele frequency in the control population deviated from the Hardy–Weinberg Equilibrium (HWE) at a p value equal or less than 0.01 were excluded from the meta-analysis. If more than one article was published by the same author using the same case series, we selected the study where the most individuals were included.

2.2. Statistical analysis

The meta-ORs were estimated using a random-effects model with the homozygous wild-type as reference group [35]. The heterogeneity was tested by Q and I^2 statistics [36]. To determine the deviation from HWE we used a publicly available program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). A visual inspection of Begg's funnel plot and Begg and Egger asymmetry tests [37] were used to investigate for publication bias when appropriate [38]. To estimate sources of heterogeneity, we conducted stratified analysis by ethnicity and study design. It was possible to perform stratified analysis by ethnicity only for the lung–*MTHFR* C677T association. Lastly, we performed sensitivity analyses on C677T by excluding studies conducted in the USA, where some common food items are regularly fortified with folate since 1998 [39].

To assess the effect modification by dietary folate intake and alcohol consumption on the association between *MTHFR* variants and cancer, we have contacted the authors of the papers that reportedly collected these variables to obtain genotype data stratified by these two covariates. In the stratified meta-analyses, subjects were classified in two classes of dietary folate intake based on the lower quartile estimated in the control population as provided by the authors, while for alcohol consumption subjects were classified as ever (current and/or former) and never drinkers. A test of heterogeneity was performed to assess for statistically significant differences among the strata estimates. Statistical analyses were carried out using the STATA software package v.9.2 (Stata Corporation, College Station, Texas) and all statistical tests are two sided.

3. Results

3.1. Studies identified

All studies included in the meta-analysis are summarized in Table 1. No overlap occurred between the studies based on case or control participation.

3.1.1. MTHFR C677T

Nine case-control studies [7,25–32] were identified for head and neck cancer, with a total of 2076 cases and 4834 controls. Among them seven were conducted on Europeans and four were population based (Table 1).

Eleven studies were identified for lung cancer [6,7,16–24,33], among them one was excluded [16] as the number of carriers of *MTHFR* 677 TT and CT was not available, and one more [33] because they partially overlapped with another study that included a larger series [7]. These 10 studies of lung cancer comprised of a total of 5274 cases and 7435 controls [6,7,17–24]. With exception of one longitudinal study [23], the others had a case-control design, among them two nested in a cohort [17,21]. Four studies were conducted on Asians and six in Europeans population, and five studies out of 10 recruited population based controls.

3.1.2. MTHFR A1298C

Four hospital-based case-control studies were identified for head and neck cancer which sums up to a total of 1439 cases and 3941 controls (Table 1) [7,27,29,30].

Table 1
Description of the studies included in the meta-analyses of the association between *MTHFR*[a] C677T and A1298C, and head and neck cancer and lung cancer

Tumour site	First author, year	No. of cases	No. of controls	Country	Source of controls	Crude OR[a] (95% CI[a])	
						677 TT *vs* CC	1298 CC *vs* AA
Head and Neck	Reljic et al. [26], 2007	81	102	Croatia	Population	0.87 (0.31–2.50)	–
	Suzuki et al. [27], 2007	237	711	Japan	Hospital	0.80 (0.52–1.25)	0.78 (0.35–1.74)
	Hung et al. [7], 2007	583	2530	Central and Eastern Europe	Hospital	1.13 (0.83–1.54)	0.87 (0.63–1.18)
	Hsiung et al. [25], 2007	278	526	USA	Population	0.66 (0.38-1.15)	–
	Vairaktaris et al. [28], 2006	110	120	Greece	Hospital	0.96 (0.32—2.95)	–
	Capaccio et al. [29], 2005	65	100	Italy	Hospital	1.56 (0.63–3.82)	1.42 (0.42–4.81)
	Neumann et al. [30], 2005	537	545	USA	Hospital	0.74 (0.47–1.17)	0.27 (0.13–0.56)
	Kureshi et al. [31], 2004	50	54	Pakistan	Population	0.16 (0.01–3.13)	–
	Weinstien et al. [32], 2002	135	146	Puerto Rico	Population	1.03 (0.47–2.27)	–
Lung	Vineis et al. [17], 2007	113	225	Europe	Population	1.43 (0.73–2.80)	–
	Suzuki et al. [18], 2007	515	1030	Japan	Hospital	0.91 (0.66–1.25)	0.92 (0.55–1.57)
	Hung et al. [7], 2007	2169	2803	Central and Eastern Europe	Hospital	1.23 (1.02–1.54)	0.86 (0.71–1.04)
	Zhang et al. [19], 2005	505	500	China	Population	1.90 (1.35–2.67)	1.75 (0.58–2.57)
	Shi et al. [6], 2005	1051	1141	USA	Hospital	0.83 (0.62–1.11)	1.38 (1.02–2.87)
	Shen M. et al. [20], 2005	122	113	China	Population	1.81 (0.81–4.03)	0.32 (0.06–1.66)
	Jeng et al. [21], 2005	59	232	Taiwan	Population	0.24 (0.03–1.92)	–
	Siemianowicz et al. [22], 2003	146	44	Poland	Hospital	3.79 (1.37–10.48)	1.78 (0.65–4.89)
	Hejmans et al. [23], 2003	44	793	The Netherlands	Population	1.07 (0.36–3.19)	–
	Shen H. et al. [24], 2001	550	554	USA	Hospital	1.02 (0.68–1.53)	1.09 (0.69–1.73)

[a] OR, Odds Ratio; CI, Confidence Interval; *MTHFR*, methylentrahydrofolate reductase.

Seven case-control studies of lung cancer reported results on *MTHFR* A1298C, comprising a total of 5098 cases and 6243 controls [6,7,18–20,22,24]. Among them two studies were population based and four out of seven conducted in European population.

3.2. Meta-analyses

3.2.1. MTHFR C677T

We did not observe an association between *MTHFR* genotype and head and neck cancer risk based on the nine studies published so far. The overall OR of head and neck cancer was 0.99 [95% confidence interval (CI): 0.81–1.22; p for heterogeneity = 0.01; I^2 = 0% (95% CI: 0–78)] and 0.92 [95% CI: 0.76–1.11; p for heterogeneity = 0.54; I^2=0% (95% CI: 0–54)] for *MTHFR* CT and TT genotypes (Fig. 1), respectively. There is no evidence of heterogeneity by study design or ethnicity. Sensitivity analysis showed an OR of 1.02 [95% CI: 0.82–1.27; p for heterogeneity = 0.68; I^2 = 0% (95% CI: 0–58)] for *MTHFR* 677 TT when excluding studies conducted in the USA [26,31].

The OR of seven out of 10 studies for lung cancer were above unity, and the meta-analysis resulted in an overall OR of 1.09 [95% CI: 0.97–1.23; p for heterogeneity = 0.09; I^2 = 40% (95% CI: 0–70)] and 1.22 [95% CI: 0.95–1.56; p for heterogeneity = 0.003; I^2 = 64% (95% CI: 11–80)] for lung cancer and *MTHFR* CT and TT genotypes (Fig. 1), respectively (p for trend = 0.001). After stratification for ethnicity, we observed OR of 1.15 (95% CI: 0.88–1.52; p for heterogeneity = 0.03; I^2 = 55% (95%CI: 0–80)) and 1.26 [95% CI: 0.70–2.50; p for heterogeneity = 0.005; I^2 = 77% (95% CI: 0–89)] for *MTHFR* 677 TT *versus* CC genotype, among Europeans and Asians, respectively (p for heterogeneity among the strata estimates of 0.85). The test for heterogeneity according to type of controls resulted in a p- value of 0.34. By excluding studies conducted in the USA, the OR of lung cancer for *MTHFR* 677 TT was 1.37 [95% CI: 1.02–1.84; p for heterogeneity = 0.015; I^2 = 60% (95% CI: 0–80), and by further excluding the study [19] showing the largest amount of *MTHFR* 677 TT individuals among controls the resulting p for heterogeneity was 0.070.

3.3. Stratified meta-analyses

We obtained *MTHFR C677T* genotype data stratified dietary folate intake and alcohol from all studies that reportedly collected these variables (three studies for head and neck [7,27,32] and three for lung [6,7,18]), and results of the stratified meta-analyses are shown in Table 2. Folate intake was collected from the studies using food frequency questionnaires, and reported as μg/day [6,18,27,32] except Hung et al. [7], who reported the number of portions/week of four foods rich in folate contents (liver, spinach, cabbage and a combination of brussels sprouts with broccoli). When stratifying data on dietary folate intake, the OR of head and neck cancer was 0.85 (95% CI: 0.63–1.16) for individuals carrying *MTHFR* 677 TT with high folate intake, while the OR was 1.37 (95% CI: 0.92–2.06) for those with low folate intake (p for heterogeneity among the two estimates = 0.06, Table 2). Similarly, stratifying lung cancer genotype data on dietary folate intake, we observed an OR of 0.94 (95% CI: 0.79–1.12) for *MTHFR* 677 TT individuals with high folate intake and an OR of 1.28 (95% CI: 0.97–1.68) for those with low folate intake (p for heterogeneity among the two estimates = 0.06, Table 2). Results of the stratified meta-analyses on alcohol consumption did not show different risk estimates for never and ever drinkers for both tumour sites (Table 2).

3.3.1. MTHFR A1298C

We did not observe an association between *MTHFR* A1298C and either head and neck cancer nor lung cancer risk. The meta-analysis of head and neck cancer provided an overall OR of 0.78 [95% CI: 0.71–1.01; p for heterogeneity = 0.09; I^2 = 59% (95% CI: 0–87)] and 0.68 [95% CI: 0.37–1.26 p for heterogeneity = 0.02; I^2 = 77% (95% CI: 0–89)] for *MTHFR* 1298 AC and CC genotypes, respectively.

The meta-analysis of lung cancer resulted in an overall OR of 0.98 [95% CI: 0.90–1.06; p for heterogeneity = 0.74; I^2 = 0% (95% CI: 0–58)] and 1.07 [95% CI: 0.83–1.38; p for heterogeneity = 0.05; I^2 = 68% (95% CI: 0–87)] for AC and CC genotypes, respectively.

4. Discussion

The meta-analysis of nine studies on head and neck cancer showed no association for either *MTHFR* C677T or A1298C genotype. The meta-analysis of 10 studies on lung cancer suggested an possible increased risk for subjects carrying the *MTHFR* 677 TT genotype, but not for *MTHFR* 1298 CC. We did however observe suggestive evidence that the effect of *MTHFR* 677 TT on both cancer sites is modified by dietary folate intake, with higher risk for cancer among *MTHFR* 677 TT individuals with low folate intake compared with those carrying the same genetic variant but with high folate levels.

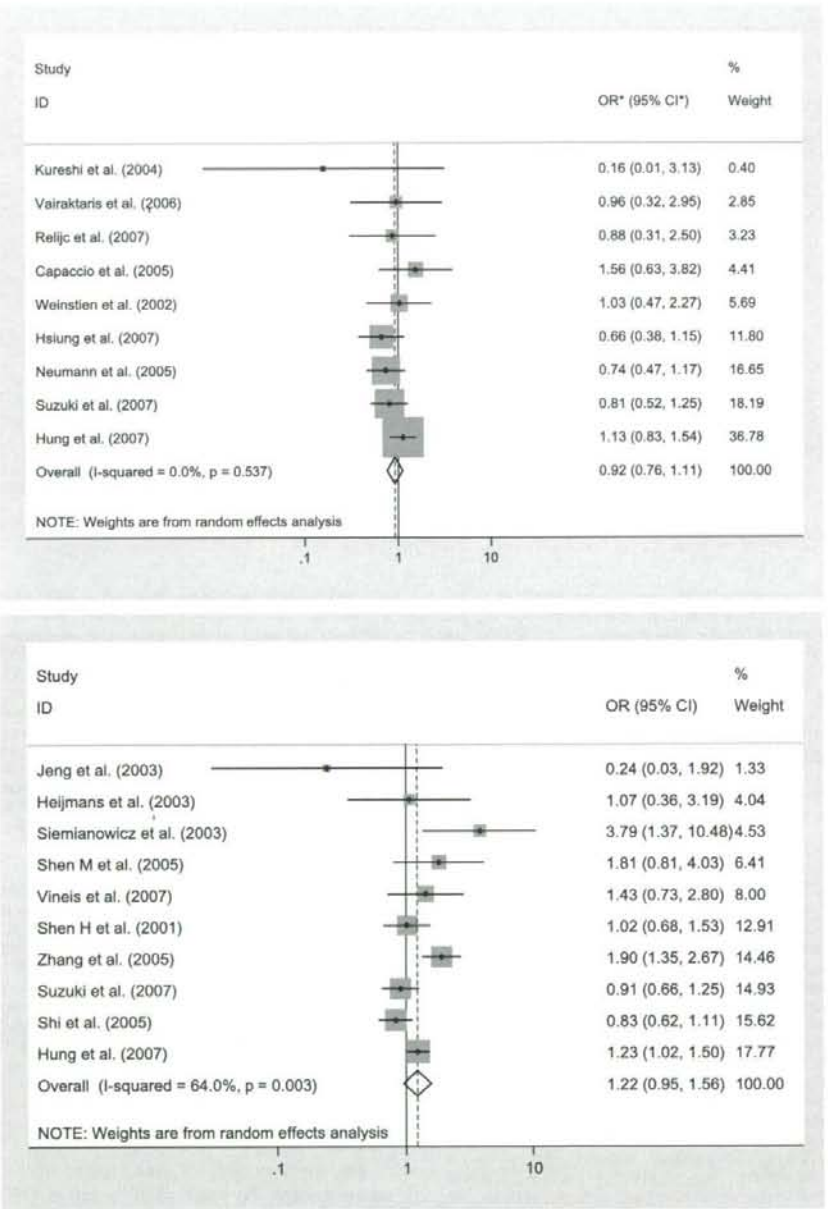

Fig. 1. Forest plot from the meta-analysis of head and neck and lung cancer risk and methylenetetrahydrofolate reductase C677T polymorphism (TT *versus* CC). *OR, Odds Ratio; CI, Confidence Interval.

These findings are in line with the hypothesis of a possible chemopreventive effect of folate in head and neck and lung carcinogenesis. Individuals carrying *MTHFR* C677T homozygous variant have lower enzyme activity, lower folate levels and subsequent aberrant DNA methylation compared with those 677 CC, and this in presence of a concomitant inadequate folate intake might be an important susceptibility factor for lung and head and neck cancer. Our results are in line with those of a recently published meta- and pooled-analysis showing that *MTHFR* 677 TT genotype is associated with an increased risk of gastric cancer, particularly among those with low folate

Table 2
Odds ratios and 95% confidence intervals from the stratified meta-analyses of the association between *MTHFR*[a] *C677T* and head and neck cancer and lung cancer

Tumour site		No. of cases	No. of controls	No. *MTHFR*[a] 677 TT cases	No. *MTHFR* 677 TT controls	OR[a,b]	95% CI[a]	*p* value for heterogeneity within strata	*p* value for heterogeneity across strata
Head and Neck[c]	Never drinkers	47	285	15	61	1.84	0.62–5.50	0.21	0.24
	Ever drinkers	517	1684	96	315	0.94	0.73–1.22	0.39	
	High folate intake	359	1127	63	247	0.85	0.63–1.16	0.72	0.06
	Low folate intake[d]	204	841	45	129	1.37	0.92–2.06	0.88	
Lung[e]	Never drinkers	279	549	49	123	0.90	0.33–2.47	0.006	0.71
	Ever drinkers	1480	2143	268	406	1.09	0.91–1.30	0.70	
	High folate intake	1447	2124	270	437	0.94	0.79–1.12	0.54	0.06
	Low folate intake[d]	718	842	132	131	1.28	0.97–1.68	0.36	

[a] OR, Odds Ratio; CI, Confidence Interval; *MTHFR*, methylenetetrahydrofolate reductase.
[b] The comparison is *MTHFR* 677 TT versus CC.
[c] Hung et al. [7], Suzuki et al. [27] and Weinstein at al. [32] studies were included.
[d] Low folate intake defined on the lower quartile estimated in the control population as provided by the authors and defined as: for head and neck <7 times/week [7], <245.7 μg/day [27] and <332.7 μg/day [32]; for lung <290.0 μg/day [6], <5 times/week [7], <253.6 μg/day [18]. See Section 3.3 for details.
[e] Shi et al. [6], Hung et al. [7] and Suzuki et al. [18] studies were included.

levels [40]. Our meta-analyses, however, failed to demonstrate overall a statistically significant risk of lung and head and neck cancer associated with *MTHFR* C677T homozygous variant genotype. Since *MTHFR* C677T appears to act as beneficial or deleterious depending on subjects' folate status, one would expect that the homozygous variant genotype would have no effect on cancer risk in population with high prevalence of folate supplement intake. More than a quarter of the weight in the results of both our meta-analyses' on lung and head and neck cancer was accounted for by studies conducted in the USA [6,24,25,30], where some common food items are regularly fortified with folate since 1998 [39]. Those studies actually showed almost the weakest association between 677 TT and lung and head and neck cancer, in fact by excluding these studies we showed a significant increased risk of lung cancer for *MTHFR* 677 TT genotype. Even if caution needs to be used when interpreting these results, both in view of the lengthy induction time for lung and head and neck cancer and the lag-time for an effect of folic acid, our results suggest a possible chemopreventive effect of folate more prominent in *MTHFR* 677 TT individuals, and a possible stronger role for the gene in those with low folate intake, which need to be addressed more in depth.

In the stratified meta-analysis according to alcohol consumption, we were unable to observe any effect modification, which is in line with the pooled analysis on gastric cancer [40]. However the information on alcohol did not take into account the amount or duration of alcohol which might be relevant especially for head and neck cancer. The present meta-analysis on *MTHFR* 677 TT and lung cancer included two additional studies [17,23] respect to the one previously published [34]. In addition, we further investigate the effect modification by folate status, which was lacking in the previous meta-analysis.

MTHFR A1298C has been reported to be in negative linkage disequilibrium with *C677T* [6,7]. The results of our meta-analyses revealed fluctuating estimates and overall null findings, which would suggest that C677T is the main *MTHFR* variant that is associated with cancer risk.

Some limitations should be considered when interpreting the results, in addition to those inherited from the meta-analysis. First, the data of the estimated dietary folate intake across the studies is collected using different food frequency questionnaires, and different cut-off values defines the lower quartile of folate intake in the studies, depending on the distribution of this variable in each specific population. Both situations, however, could lead to non-differential misclassification of the exposure and biased effect measures toward the null. If such bias is present in our data, it would indicate that the underlying true effect modification should be stronger than what we observed. Second, the subgroup meta-analyses on folate intake and alcohol consumption are based on a small number of studies with such information available. Nevertheless the total number of subjects included in this part of the analysis comprise the largest sample size so far.

Despite all these remarks, the observed increased risk for lung cancer among *MTHFR* 677 homozygous variant carriers with low dietary folate intake suggests that dietary folate might be protective in carcinogenesis especially in situation of impaired folate status as recently shown for gastric cancer [40]. Since more than half of the included studies were based on a limited number of cases (<200) it is critical that larger prospective studies, collecting detailed lifestyle habits data and repeated serological dosage of folate levels, are performed, in order to clarify the preventive role of folate in tobacco- and alcohol-related cancers. To overcome the limitation of meta-analysis, a coordinated genotyping of *MTHFR C677T* is now underway in the International Lung Cancer Consortium (http://ilcco.iarc.fr/), which will allow us to investigate the role of *MTHFR* in lung carcinogenesis and its potential effect modification by folate consumption.

Conflict of interest statement

Authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence their work.

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Downregulation of *EphA1* in colorectal carcinomas correlates with invasion and metastasis

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The Eph gene family has important roles in the developmental processes and may also be involved in the initiation, progression, and metastasis of certain types of cancers. In the present study, quantitative real-time reverse-transcriptase PCR was performed to detect the expression of *EphA1* transcript in 5 colon cancer cell lines and 75 colorectal carcinomas. Immunohistochemical staining was used to check the expression of *EphA1* protein in 20 colorectal adenomas and in 111 colorectal carcinomas specimens. *EphA1* protein expression was not completely consistent with transcript expression. *EphA1* protein was expressed in all adenomas and reduced in 54% colorectal cancers. Reduced expression of *EphA1* protein occurred more often in male patients ($P=0.028$) and in patients with poor differentiation ($P=0.027$), greater depth of wall invasion ($P=0.003$), lymph node metastasis ($P=0.034$), and advanced tumor stage ($P=0.003$). Patients with reduced *EphA1* expression had a poor overall survival ($P=0.059$). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively). Our data indicate that the *EphA1* may play different roles during the different stages of colorectal carcinoma progression.
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Keywords: *EphA1*; colorectal carcinoma; invasion; metastasis; survival

Colorectal carcinoma is one of the most common malignant tumors. There are estimated 112 340 new cases of colon cancer and 41 420 new cases of rectum cancer in USA, and estimated 52 180 cases of colorectal cancer patients died in 2007. The incidence and mortality rate of colorectal cancer rank the third in all types of cancers both of male and female patients.[1] The search for new molecular targets of early diagnosis, rational therapy, and prognosis is the current research hot spot.

Eph receptors, the largest subfamily of the receptor tyrosine kinases, are divided into two subfamilies, EphA and EphB, based on the sequence homology of their extracellular domains and their affinity to bind corresponding ligands, EphrinA and EphrinB (Eph Nomenclature Committee, 1997).[2] The Eph family of receptor tyrosine kinases has important roles in diverse biological processes including nervous system development,[3–5] angiogenesis,[6] and vascular system development.[7] A number of Eph receptors and their ephrins ligands are implicated in carcinogenesis.[8–10] *EphA1*, the first member of Eph receptors tyrosine kinase, was isolated from erythropoietin-producing hepatocellular carcinoma cell lines and is located on chromosome 7q34.[17] It is widely expressed in normal tissues including lung, small intestinal, kidney, bladder, thymus, and colon.[18] The expression level of *EphA1* in human cancers is variable. Overexpression of *EphA1* was observed in certain types of tumors including ovarian carcinoma,[19] and head and neck squamous carcinoma.[20] Reduced expression of *EphA1* was detected in prostate cancer cell lines,[21] breast carcinoma cell lines,[12] and basal-cell carcinomas and squamous-cell carcinoma specimens of the skin.[22] There was a marginal study of *EphA1*

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expression in colorectal carcinoma specimens, particularly, with respect to clinicopathologic parameters. In this study, we performed quantitative real-time reverse-transcriptase PCR (RT-PCR) and immunohistochemistry to detect the expression of *EphA1* mRNA and protein in a set of colorectal carcinomas and adenomas. And the association of *EphA1* expression levels with clinicopathologic parameters of colorectal carcinomas was analyzed. To our knowledge, this is the first description of the role of *EphA1* in colorectal progression and prognosis.

Materials and methods

Five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 were used in the present study. The cells were routinely cultured in Dulbecco's modified Eagle's medium (NISSUI Pharmaceutical Co., Tokyo, Japan), supplemented with 1 mmol/l L-glutamine, 10% fetal bovine serum (FBS; Life Technologies Inc.), and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

All the tissue samples in our study were collected from 111 patients with colorectal carcinoma and 20 patients with colorectal adenomas, as part of a study approved by the Research Ethics Board of the Nanjing Jinling Hospital, Clinical School of Medical College of Nanjing University. These patients had undergone surgery in Nanjing Jinling Hospital between 2004 and 2006 without any preoperative therapy. Among the 111 cases of colorectal carcinomas, 75 fresh tissue samples including cancer tissues and matched normal mucosas were immediately frozen in liquid nitrogen after the resection and then stored at −80°C for the preparation of the total RNA. Formalin-fixed and paraffin-embedded tumor tissues were sectioned at 4 μm thickness and stained with hematoxylin and eosin for the pathological identification. The patients included 66 men and 45 women. Ages ranged from 23 to 84 (median age: 59 years). The distribution of the tumors by sites of origin was as follows: the cecum and ascending colon, 26 tumors; the transverse colon, 4 tumors; the sigmoid colon, 14 tumors; and the rectum, 67 tumors. The clinicopathologic variables of the 111 patients of colorectal carcinoma were shown in Table 1. The tumor stage was classified according to the TNM classification of World Health Organization of 2007.

Quantitative Real-Time RT-PCR

The total RNA was extracted using the RNA extraction reagent TRIzol (Invitrogen, CA, USA) according to the manufacturer's protocol. Single-strand cDNA was synthesized using 2 μg total RNA with an oligo(dT) primer. Quantitative real-time RT-PCR was performed to detect the *EphA1* transcript expression in colorectal carcinoma on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). The experiments were run in triplicate. The sense primer, antisense primer, and TaqMan probe for detection of *EphA1* were designed according to the *EphA1* mRNA sequence (GenBank accession number: NM_005232). The sense primer is 5′-ATCTTTGGGCTGCTGCTTGG-3′ and the antisense primer is 5′-GCTTGTCCTCTCGATCCACA TC-3′. The PCR products are 127 bp long. The TaqMan probe is 5′-(FAM) CGGTCACGCTGC CTCTGCTGCC (Eclipse)-3′. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control (GenBank accession number: NM_002046). The sense primer is 5′-CCAGGTGGTCTCCTCTGACTT-3′ and the antisense primer is 5′-GTTGCTGTAGCCAAATTCGTT GT-3′. The PCR products are 130 bp long. The probe is 5′-(FAM) AACAGCGACACCCACTCCTCCACC (Eclipse)-3′. The values of *EphA1* mRNA expression were normalized using the *GAPDH* expression. The primers and probes for *EphA1* and *GAPDH* were synthesized by TaKaRa Biotechnology Inc. (Dalian, China).

The reaction mixture consisted of 3.0 μl 10 × buffer; 3.0 μl 2.0 μmol/l deoxy-ribonucleoside triphosphates (dNTPs; Invitrogen); 3.0 μl 3.0 μmol/l sense primer; 3.0 μl 3.0 μmol/l antisense primer; 1.0 μl 3.0 μmol/l fluorescence probe; 0.20 μl 5 U/μl Takara ExTaq Hotstart Taq (TaKaRa Biotechnology), 0.6 μM 5-carboxy-X-rhodamine reference dye (Invitrogen), 2.0 μl cDNA template, and distilled water for a total volume of 30 μl. The PCR cycling conditions were used as follows: 2 min at 95°C, followed by 40 amplification cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 1 min.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 2 μm thickness. All the sections were deparaffinized using xylene, dehydrated by gradient ethanol, and then rehydrated with deionized water. Heat-mediated antigen retrieval was run by autoclave treatment (120°C for 2 min in 1 mmol/l EDTA, pH 8.0) and then followed by cooling at room temperature. Incubation with a polyclonal antibody raised against the COOH terminus of the human *EphA1* receptor (dilution 1:100, ABGENT, San Diego, USA) was performed overnight at 4°C. After washing with phosphate-buffered saline (pH 7.4), the sections were then incubated with secondary antibody (Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3′-diaminobenzidine. Nuclei were counterstained with hematoxylin.

Table 1 Correlation between expression of EphA1 transcript and clinicopathologic parameters in 75 colorectal carcinomas

	Case number	*N/T>2*	*N/T0.5-2*	*N/T<0.5*	P-*value*
Overall	75	28	17	30	—
Sex					
Male	44	17	10	17	0.952
Female	31	11	7	13	
Age (years)					
≤55	36	13	8	15	0.96
>55	39	15	9	15	
Location					
Rectum+sigmoid colon	57	23	13	21	0.556
Colon	18	5	4	9	
Tumor size (cm)					
≤5	49	18	11	20	0.98
>5	26	10	6	10	
Depth of wall invasion					
Mucosa+submucosa	4	3	1	0	0.278
Muscularis propria	19	7	2	10	
Subserosa+serosa	52	18	14	20	
Tumor differentiation					
Well+moderate	48	15	13	20	0.36
Poor+mucinous	27	13	4	10	
Lymph node metastasis					
Negative	46	18	9	19	0.72
Positive	29	10	8	11	
Clinical stage (TNM)					
I	20	9	2	9	0.617
II	25	8	7	10	
III+IV	30	11	8	11	
Stage (Dukes)					
A+B	45	17	9	19	0.78
C	30	11	8	11	

N/T, normal–tumor; TNM, tumor, node, metastasis.

The immunostaining results were evaluated independently by three pathologists. The different results were unified by consensus. The score of *EphA1* expression was made semiquantitatively by assessing the percentage of stained cells and the staining intensity in both tumor tissue and normal mucosa. The percentage of positive cells was rated as follows: 0 score for 0–5%, 1 score for 6–25%, 2 scores for 26–50%, and 3 scores for more than 50%. The staining intensity was rated as follows: 0 score for no staining, 1 score for weak staining, 2 scores for moderate staining, and 3 scores for strong staining. The scores from the percentage and intensity were added to an overall score. The expression of the *EphA1* protein in colorectal carcinomas was categorized into downregulation, upregulation, and no difference by comparing the overall score in tumor tissue *vs* matched normal mucosa.

Methylation-Specific PCR

Genomic DNA was modified by sodium bisulfite, as described by Clark *et al*.[23] Primers were designed to discriminate between methylated and unmethylated alleles after sodium bisulfite treatment. Primer sequences were chosen for the regions containing frequent CpG near the 3′-end of the primers to provide maximum discrimination between methylated and unmethylated DNA. Aliquots (2 μl) were amplified in a 30-μl reaction mixture consisting of 1 × buffer (10 mM Tris-HCl, 2.0 mM $MgCl_2$, 50 mM KCl, pH 8.3), 1 U Takara ExTaq Hotstartaq, 260 μM dNTPs, and 0.3 μM of the primer sets. The PCR reaction involved 2 min at 95°C, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and finally 10 min at 72°C. The methylation-specific primers were 5′-ATTCGGGTTATTGTTTTAGGTTTC-3′ (forward) and 5′-GAAAATCGATACCTTCCTTAACG-3′ (reverse). The PCR products were 129-bp long. Unmethylation-specific primers were 5′-ATTTGGGTTATTGTTTTAGGTTTTG-3′ (forward) and 5′-ACAAAAATCAATACCTTCCTTAACAC-3′ (reverse). Primer sets for detection of methylated and unmethylated DNA were located at the same sites of genomic sequence (forward primer was located at −35 to −12 from translation start site; reverse primer was located at 71–93; Figure 4). The

PCR products were 131-bp long. The PCR products were separated on 8% nondenaturing polyacrylamide gel, followed by ethidium-bromide staining.

Statistical Analysis

The χ^2-test was adopted to determine differences among intergroup variables by use of SPSS 15.0. software (SPSS, Chicago, IL, USA). Kaplan–Meier survival analysis was used to examine the relationship between categorical groups and survival for univariate analysis. A P-value <0.05 was considered statistically significant.

Results

Expression of the *EphA1* Transcript in Colon Cancer Cell Lines and in Colorectal Carcinomas

RT-PCR was performed to detect the expression of *EphA1* transcript in colon cancer cell lines DLD1, HT29, HCT116, SW480, and SW620. *EphA1* mRNA was detected in all the colon cancer cell lines (Figure 1). Quantitative real-time RT-PCR was used to detect the expression of *EphA1* transcript in 75 fresh specimens of colorectal carcinomas. The ratio of normal–tumor (N/T) was based on the relative expression level of *EphA1* transcript in paired normal mucosa and the tumor tissues of the same patient. The results were classified into three groups according to the ratio of the two: downregulation (N/T > 2), upregulation (N/T < 0.5), and no difference (N/T 0.5–2). Down and upregulation of *EphA1* transcript were observed in 37% (28 of 75) and 40% (30 of 75) of the specimens, respectively (Table 1). The association of expression of *EphA1* transcript with the sex, age, tumor site, size, depth of wall invasion, differentiation, clinical stage, lymph node metastasis, and Dukes stage were analyzed. No any significant difference between the *EphA1* transcript expression and these clinicopathologic parameters was found. The data were summarized in Table 1.

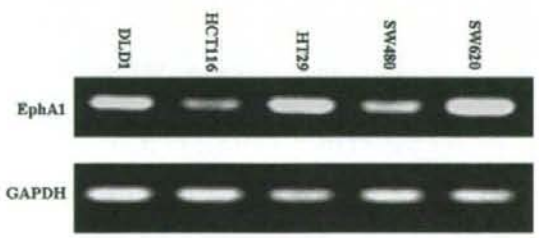

Figure 1 Expression of *EphA1* in colon cancer cell lines DLD1, HCT116, HT29, SW480, SW620. Housekeeping gene *GAPDH* was used as an internal control.

Expression of *EphA1* Protein in Colorectal Carcinomas and Adenomas

The *EphA1* protein was detected in most of the normal mucosa cells (Figure 2a①). The adenoma cells expressed *EphA1* protein diffusely (Figure 2a②). A heterogeneous *EphA1*-staining pattern between cells was observed in carcinoma tissue sections. The up and downregulations of the *EphA1* protein were observed in 31% (34 of 111) and 54% (60 of 111) cases of colorectal carcinomas, respectively (Table 2; Figure 2a③④). The immunostaining of *EphA1* was observed as particles in cytoplasm or distributed homogenously in cytoplasm (Figure 2b⑤⑥).

The Significance of Reduced Expression of the *EphA1* Protein in Colorectal Carcinoma

The expression of *EphA1* protein was significantly related to sex, depth of wall invasion, differentiation, lymphatic metastasis, and clinical stage. The reduced expression of *EphA1* was more often occurred in poorly differentiated colorectal carcinomas and mucinous adenocarcinomas than in well- and moderately differentiated cases ($P=0.027$). The patients with reduced *EphA1* protein had deeper serosa and subserosa invasiveness than those without *EphA1* downregulation ($P=0.003$). Colorectal carcinomas with reduced *EphA1* expression had more advanced tumor stage ($P=0.003$) and lymph node metastasis ($P=0.034$). Reduced expression of the *EphA1* protein was more often detected in male than in female patients ($P=0.028$). There was no significant association with other clinicopathologic variables (Table 2).

Reduced *EphA1* Protein Expression is Associated with Poor Survival in Patients with Colorectal Carcinoma

We examined the association of *EphA1* protein expression with clinical outcome. The Kaplan–Meier survival analysis showed patients with reduced *EphA1* expression had shorter survival than those with high *EphA1* expression (log-rank test, $P=0.059$; Figure 3①). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively; Figure 3②③).

Detection of Methylated *EphA1* DNA in Colon Cancer Cell Lines

Five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 were checked for methylation status at promoter-associated region of *EphA1* by methylation-specific PCR. Methylated DNA of *EphA1* was detected in all five tested colon cancer cell lines (Figure 4).

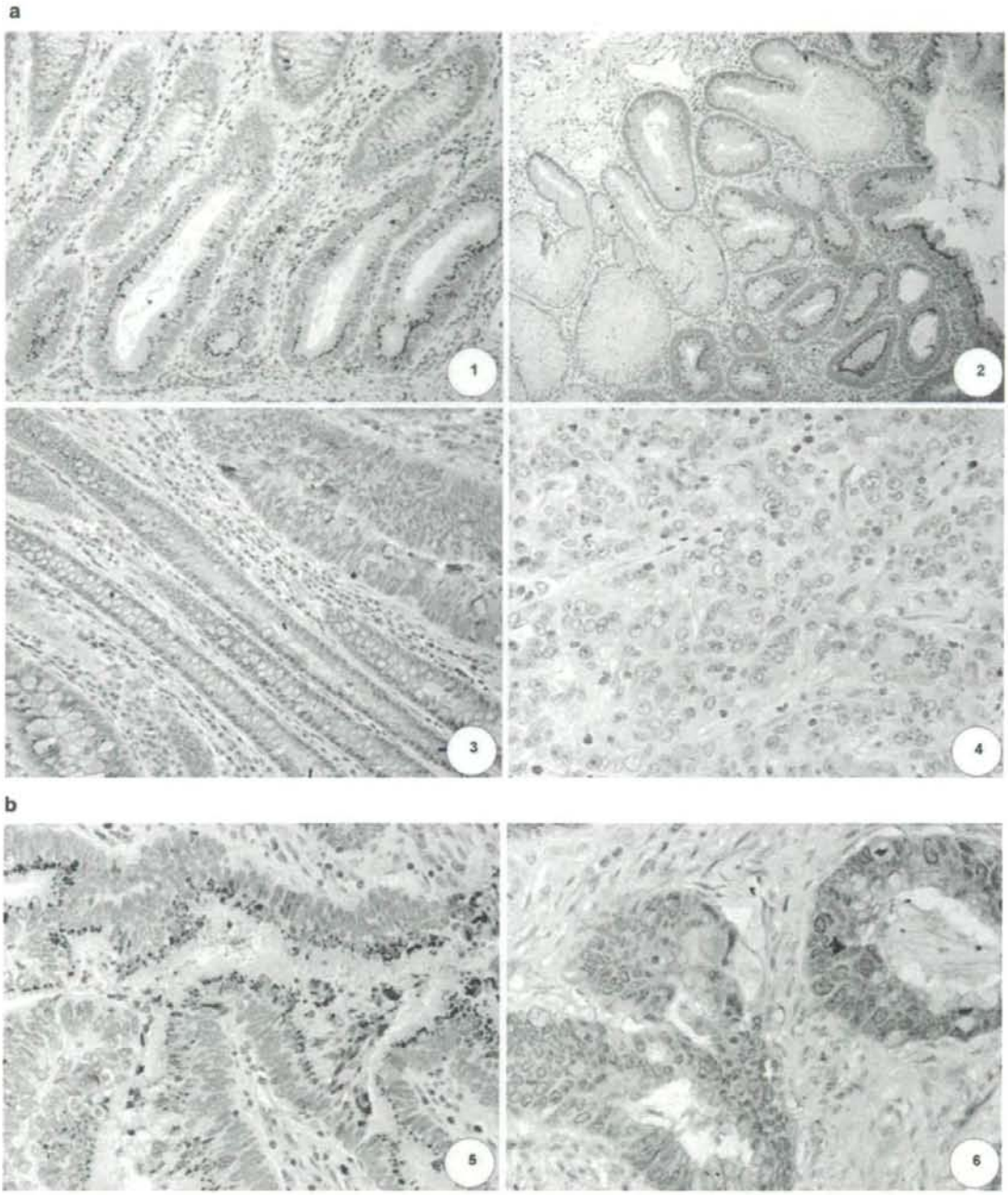

Figure 2 (**a**) Immunohistochemical staining of *EphA1* in colorectal carcinomas. ① The expression of *EphA1* in normal mucosa (EnVision, original magnification ×200). ② Upregulation of *EphA1* in adenoma cells (right lower) compared with normal mucosas (left upper; EnVision, original magnification ×100). ③ Downregulation of *EphA1* in carcinoma cells (right upper) compared with normal mucosas (left lower; EnVision, original magnification ×200). ④ Lost expression of *EphA1* in poorly differentiated carcinoma cells (EnVision, original magnification ×400). (**b**) ⑤ Strong immunoreactivity was detected in moderately differentiated carcinoma cells. The subcellular localization of *EphA1* protein revealed the accentuation of golgiosome (EnVision, original magnification ×400). ⑥ *EphA1* protein was expressed homogeneously in cytoplasm (EnVision, original magnification ×400).

Table 2 Correlation between EphA1 protein expression and clinicopathologic parameters in 111 colorectal carcinomas

	Case number	*Downregulation*	*No difference*	*Upregulation*	P-value
Overall	111	60	17	34	—
Sex					
Male	66	37	14	15	0.028
Female	45	23	3	19	
Age (years)					
≤55	49	31	8	10	0.109
>55	62	29	9	24	
Location					
Rectum+sigmoid colon	81	40	12	29	0.144
Colon	30	20	5	5	
Tumor size (cm)					
≤5	80	40	14	26	0.352
>5	31	20	3	8	
Depth of wall invasion					
Mucosa+submucosa	10	3	3	4	0.003
Muscularis propria	24	7	3	14	
Subserosa+serosa	77	50	11	16	
Tumor differentiation					
Well+moderate	80	37	15	28	0.027
Poor+mucinous	31	23	2	6	
Lymphatic metastases					
Negative	66	29	12	25	0.034
Positive	45	31	5	9	
Clinical stage (TNM)					
I	28	7	5	16	0.003
II	37	22	7	8	
III+IV	46	31	5	10	
Stage (Dukes)					
A+B	65	29	12	24	0.06
C	46	31	5	10	

N/T, normal–tumor; TNM, tumor, node, metastasis.

Discussion

The Eph gene family is the largest subfamily of receptor tyrosine kinase including at least 16 receptors and 9 ligands. The role of Eph family in developing processes has been well documented. The interaction of Eph receptors and their ligands controls the cells repulsion and movement during tissue patterning in embryonic development. However, the roles of Eph and Ephrin proteins in tumorigenesis are not clearly established. The Eph family was initially known as a putative oncogene based on their overexpression in certain types of human cancers. The *EphA2* receptor is overexpressed in colorectal,[24] gastric,[25] ovarian,[11] and esophageal squamous-cell carcinoma.[26] The *EphA4* receptor is overexpressed in pancreatic ductal adenocarcinoma,[27] and *Ephrin B1* is overexpressed in ovarian carcinoma.[28] More recently, increasing data have shown that some members of Eph receptors and Ephrin ligands have roles of tumor suppressor. A representative example is that certain EphB subfamily proteins, including *EphB2*, *EphB4*, suppress colorectal cancer progression through Wnt signal pathway.[9,10,29]

The *EphA1* receptor is widely expressed in human normal tissues. However, its expression levels in different types of human tumors are greatly diverse, and its role in tumorigenesis is still very vague. In the present study, we described the expression of the *EphA1* transcript and protein in colorectal carcinomas and analyzed the association of expression of *EphA1* with clinicopathologic parameters.

The *EphA1* transcript was detected in all five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 by RT-PCR (Figure 1). The expression levels of *EphA1* mRNA in five colon cancer cell lines were different. There is a CG-rich region around *EphA1* translation start site (Figure 4a), the methylation, and unmethylation-specific primer sets were designed by using web software MethPrimer (http://www.urogene.org//methprimer/). Methylated and unmethylated DNA

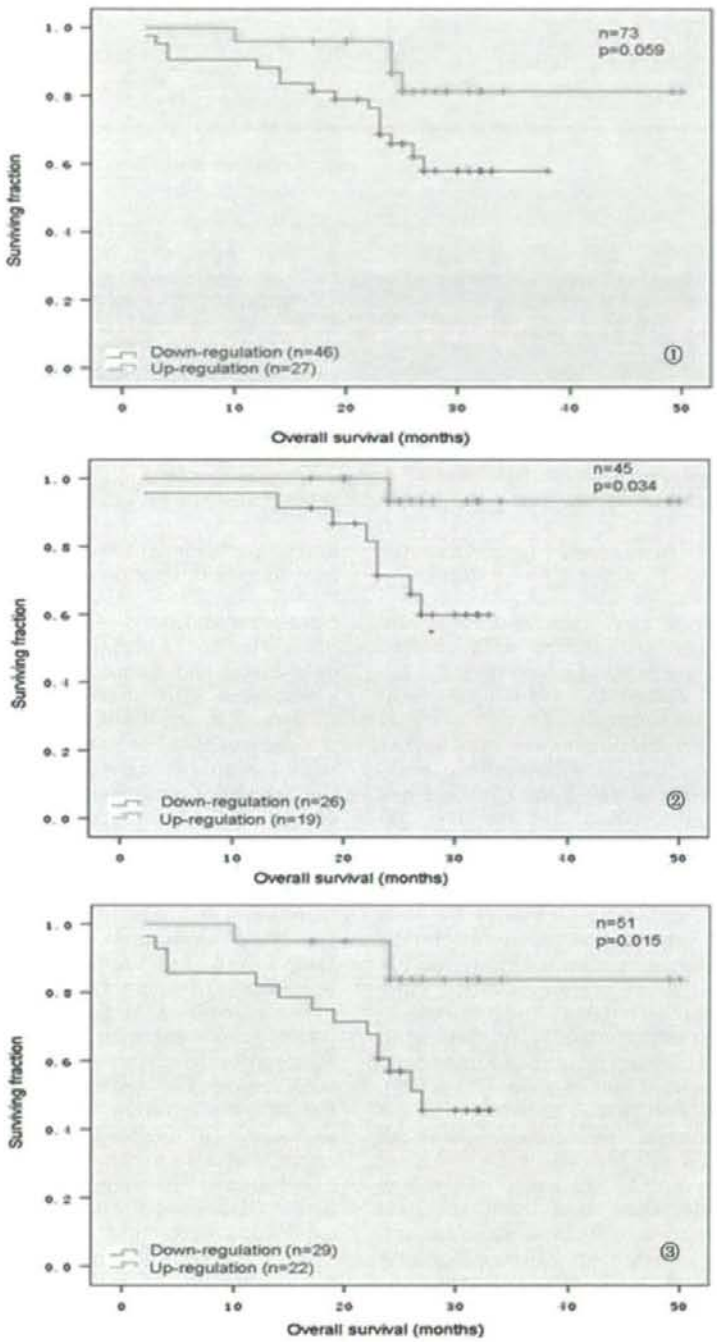

Figure 3 ① Kaplan–Meier plots of overall survival showed patients with *EphA1* downregulation had a shorter survival than those with *EphA1* upregulation ($P=0.059$). ②③ Kaplan–Meier plots of overall survival showed reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively).

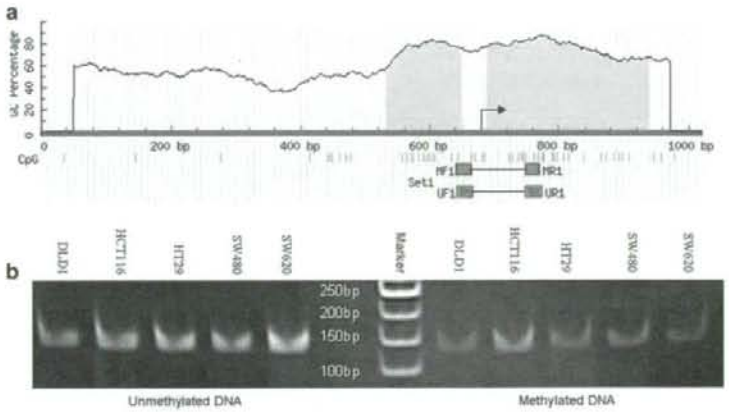

Figure 4 (**a**) Schematic show of the promoter-associated CpG island in *EphA1* and the location of PCR primer sets for specific detection of methylation and unmethylation *EphA1* DNA. The arrow showed the translation start site of *EphA1*. (**b**) Methylated and unmethylated DNAs of *EphA1* were detected in colon cancer cell lines.

of *EphA1* was detected in all tested five colon cancer cell lines (Figure 4b). To explore the methylation status of the promoter-associated CpG island of *EphA1* in tissue DNA of colorectal carcinomas and the association of the methylation with clinicopathologic parameters will be our next project.

The expression of *EphA1* transcript was downregulated in 37% and upregulated in 40% tested samples. No significant relation between the *EphA1* transcript expression and clinicopathologic parameters was found (Table 1). The *EphA1* protein was detected in most of the normal mucosa cells and diffusely expressed in adenoma cells (Figure 2). However, in the colorectal carcinomas, the *EphA1* expression was showed heterogeneity in carcinoma cells both of intra- and inter-samples. Our data suggest that *EphA1* protein was partly lost in the transition from adenomas to adenocarcinomas. Reduced expression of *EphA1* protein occurred more often in male patients ($P=0.028$) and in patients with poor differentiation ($P=0.027$), greater depth of wall invasion ($P=0.003$), lymph node metastasis ($P=0.034$), and advanced tumor stage ($P=0.003$). These data show that *EphA1* may be involved in the progression of colorectal carcinomas. Although *EphA1* has a closer homologous sequence and more similar structure to *EphA2* than any other Eph receptor,[30] our results show that there are great different features between them in terms of protein expression and its relation to clinicopathologic parameters in colorectal carcinomas. *EphA2* expression is present at the cytomembrane of the normal colorectal epithelium. However, *EphA2* immunoreactivity in colorectal carcinoma cells was diffusely distributed throughout the cytoplasm, with little staining of the cytomembrane.[24] In this study, different subcellular localization of EphA1 was found. In normal mucosa gland and adenoma cells, the immunostaining of EphA1 was showed dense brown particles in the Golgi's body. This staining pattern was only showed in parts of well-differentiated carcinoma cells. In poorly differentiated carcinoma and parts of well-differentiated carcinoma cells, the EphA1 was diffusely stained in cytoplasm. The staining pattern of EphA1 in skin ulcers was also altered, in which EphA1 was expressed in keratinocytes adjacent to the rim of the ulcer with an intense cytoplasmic staining, but with a membranous staining in those distant from the rim of the same ulcer.[22] The mechanism for this altered EphA1 immunostaining pattern is unknown. In addition, overexpression of *EphA2* is associated with metastasis and stage of the cancer.[24] However, in this study, reduced expression of *EphA1* occurred more often in patients with advanced tumor stage and lymph node metastasis. Our data suggest that *EphA1* and *EphA2* play different roles in the progression of colorectal carcinomas.

The expression of *EphA1* protein was not completely consistent with the transcript expression in 75 samples, in which only 35% (26 of 75) showed consistency. This pattern was also reported on *EphB4* expression in breast carcinomas,[31] *EphA2* expression in bladder carcinomas,[32] and *EphA7* expression in hepatocellular carcinomas.[18] The post-transcription regulation mechanisms interpreted *EphB4* and *EphA2* differential expression between protein and transcript. Upregulation of *EphA7* mRNA in hepatocellular carcinomas may be attributable to higher vascularization in the investigated tumor, resulting in intercellulalr cross-contaminations. In the present study, *EphA1* staining was not observed in stromal cells, vascular endothelial cells, or lymphocytes; we postulate that post-transcription, post-translation regulation mechanisms, or quick degradation of unstable *EphA1* protein are the reasons of inconsistent expression of *EphA1* mRNA and protein.

Follow-up information was available in 73 patients with a follow-up duration of 2–50 months (median time: 25 months), including 46 cases with reduced *EphA1* expression and 27 cases with *EphA1* upregulation. The overall survival rate in patients with *EphA1* downregulation was shorter than that in patients with *EphA1* upregulation (log-rank test, $P=0.059$). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively). The protective roles of *EphA1* protein in aged colorectal patients and patients with rectal cancers and sigmoid colon cancers are more obvious.

In summary, reduced expression of the *EphA1* protein in colorectal carcinomas is related to invasiveness, differentiation, metastasis, stage, and prognosis. Our data implicate that *EphA1* receptor may play different roles in the different stage of progression of colorectal carcinomas.

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Long-Term Outcome of Esophageal Mucosal Squamous Cell Carcinoma Without Lymphovascular Involvement After Endoscopic Resection

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BACKGROUND. Esophageal cancer is an aggressive cancer with a reported 3-year survival of 20%. However, early-stage esophageal cancer can be cured by endoscopic resection (ER). The long-term survival of esophageal mucosal squamous cell carcinoma after ER was investigated by calculating the standard mortality rate (SMR).

METHODS. From January 1995 to December 2004, 110 patients with 138 esophageal mucosal squamous cell carcinomas without lymphovascular involvement were treated by ER. Long-term survival after ER was compared with that in the general population by calculating SMR. Subgroup analysis of patients without second primary cancer diagnosed within 1 year before ER (subgroup A) was also performed.

RESULTS. A total of 108 patients (98.2%) were followed-up completely, with a mean observation period of 4.7 (0.4–11.3) years. The cumulative 5-year survival rate of all patients and subgroup A was 79.5% and 86.6%, respectively. Overall mortality (SMR, 1.68; 95% confidence interval [CI], 1.05–2.55) and mortality from malignant tumor (SMR, 3.14; 95% CI, 1.79–5.09) was significantly higher than that in the general population. SMR of esophageal cancer was high, although it was not significantly different from that in the general population (SMR, 4.82; 95% CI, 0.06–26.81). In subgroup A overall mortality (SMR, 0.86; 95% CI, 0.41–1.57) was similar to that in the general population.

CONCLUSIONS. High overall mortality in patients with esophageal mucosal cancer after ER was mainly due to elevated mortality from second primary cancer. Favorable mortality in subgroup A indicates the efficiency of ER as a curative treatment for esophageal mucosal cancer. ***Cancer* 2008;112:2166–72.** © *2008 American Cancer Society.*

KEYWORDS: esophageal neoplasms/surgery, esophagoscopy, carcinoma, squamous cell/therapy, treatment outcome, survival analysis.

Esophageal cancer is the fifth leading cause of death from cancer.[1] Squamous cell carcinoma is the prevailing tumor type in Asia, whereas this has now been overtaken by esophageal adenocarcinoma in Europe and North America.[2] Localized esophageal cancer, regardless of its histologic type, is commonly treated with surgical resection or chemoradiation, or their combination.[2–4] Even after aggressive therapy the 3-year survival rate of patients with local-regional esophageal cancer is 20%.[5,6] Moreover, surgical resection and chemoradiation are associated with high treatment-related morbidity and mortality.[2,4] Thus, advanced-stage esophageal cancer is a treatable but rarely curable disease.

Informed consent was obtained from all patients before endoscopic resection was performed.

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However, this cancer, when diagnosed at an early stage, can be cured by endoscopic resection (ER).[2,7–9] Since the 1990s, many early esophageal cancers have been treated by ER in Japan. More recently, in Western countries ER of gastrointestinal lesions, especially Barrett esophagus, with high-grade dysplasia or cancer has been increasingly used.[10,11] The techniques of ER are designed to completely remove the diseased mucosa by resection through the middle or deeper part of the submucosa. Then the resected specimen is used for pathologic staging to stratify the patient's risk of developing metastasis. Although this method is widely used in Japan, there are relatively few reports on long-term survival after ER.[7–9] For further development of this method, investigation of long-term survival and confirming its adequacy is important.

In the assessment of survival, comparison of mortality rates between the study subjects and the general population are sometimes used to assess the excess mortality risk in the study subjects. The ratio of actual observed mortality in the study group and the expected mortality calculated from the general population is known as the standardized mortality ratio (SMR). In this study we assessed the efficacy of ER for mucosal esophageal cancer by obtaining detailed follow-up data from patients after ER and calculating SMR.

MATERIALS AND METHODS

Patients

From January 1995 to December 2004, all patients with esophageal cancer were registered, along with details including therapeutic modalities, with the hospital cancer registry in Osaka Medical Center for Cancer and Cardiovascular Diseases, Japan. Data about cancer spread and treatment strategy were obtained from this database.

Detailed clinicopathologic data of patients who underwent ER were collected from medical records and included the location, size, and type of tumor and the histologic findings of the resected specimens. From these datasets we identified 110 patients with 138 esophageal cancers who met our inclusion criteria: 1) histologically proven squamous cell carcinoma; 2) mucosal cancer, including in situ lesions, without lymphovascular involvement; 3) no lymph node or distant metastasis; and 4) no previous history of radiotherapy, surgery, or ER for esophageal cancer.

ER

Before ER, endoscopic examination with chromoendoscopy after direct instillation of 1.5% iodine solution was performed in all patients to evaluate the depth and lateral spread of cancer. Computed tomography of the chest and abdomen was also carried out to identify lymph node metastasis. Lymph nodes $\geq$10 mm in diameter were defined as metastatic.

ER was indicated for esophageal cancer without ulcer or obvious protrusion that suggested invasion to a deeper part of the submucosal layer. ER was limited to lesions $\leq$50 mm in diameter. Lesions >50 mm were resected only upon patient request. Circumferential resections were avoided because of the risk of stricture formation. ER was performed under intravenous sedation with midazolam and pentazocine.

ER was performed by the endoscopic mucosal resection (EMR) method or the endoscopic submucosal dissection method. EMR was mainly performed using the standard strip biopsy method,[12] with a 2-channel endoscope (GIF-2T200 or 2T-240; Olympus, Tokyo, Japan). Saline was injected into the submucosa beneath the lesion with an injection needle (Varixor 23G; Top, Tokyo, Japan). A high-frequency electrosurgical snare and grasping forceps were passed through the channel. An area near the lesion was grasped with the forceps to capture the lesion into the snare and the lesion was strangulated. Forced coagulation current was then applied to resect the lesion.

Endoscopic submucosal dissection was mainly performed using a Hook knife as described by Oyama et al.[13] A forward-viewing endoscope (GIF-Q240Z; Olympus) with an attachment (D-201-11,804; Olympus) on its tip was introduced. Marking dots were made 2–3 mm outside the margins of the lesion with a Hook knife. A 200 mL 10% glycerin solution with 1 mL 0.1% adrenaline was injected into the submucosa and the mucosa was incised outside the marking dots with the Hook knife using the Endocut mode of an electrosurgical generator (ICC 200; Erbe, Tubingen, Germany). The diseased mucosa was then separated from the muscularis propria by the injection of glycerin solution into the submucosa. The submucosal connective tissue just beneath the lesion was dissected from the muscle layer using the Hook knife in the forced coagulation mode. Injection was repeated as needed, and further resection was carried out to ensure total removal of the lesion.

Complete local remission was defined as the disappearance of iodine-unstained lesions on endoscopic examination and no evidence of residual cancer on biopsy specimens at first follow-up. Informed consent was obtained from all patients before ER was performed.

Histologic Evaluation and Further Treatment

All specimens were cut into 2-mm slices, embedded in paraffin, and subjected to hematoxylin and eosin

staining. Detailed histologic assessment was performed according to the Japanese Classification of Esophageal Carcinoma.[14] Mucosal cancer without lymphovascular involvement was followed up without any additional treatment immediately after ER. In patients with lymphovascular involvement and/or submucosal invasion the risk of metastasis was estimated based on the histologic findings. Further treatment was decided on depending on the risk of metastasis and the patient's condition.

Follow-up

After ER all patients were included in a follow-up program. Upper gastrointestinal endoscopy with iodine staining was conducted at 3 and 9 months and annually thereafter. Computed tomography of the neck, chest, and abdomen was performed at least once a year to detect lymph node or distant metastases.

Follow-up patient data were obtained by referral to their medical records. Causes of death were confirmed by death certificates documented by the patients' physicians. For the outcome of patients who moved away from our hospital we attempted to obtain details by questionnaires or telephone conversations with their families and referring physicians. The International Classification of Diseases, Ninth Revision, Clinical Modification was used to code and classify mortality data. The starting date of the follow-up was defined as the date of ER and the end of the follow-up was either the date of death or the end of December 2006, whichever occurred first. Patients with unknown vital status were regarded as censored at the time when they were last known to be alive.

Subgroup Analysis

This study cohort included many patients with multiple primary cancers. We defined synchronous or metachronous (including previous) cancers other than esophageal as second primary cancers. In patients with multiple primary cancers, second primary cancers largely influenced the clinical course after ER. In a patient receiving ER, we usually obtained information about second primary cancer from medical records and interviews with the patient and a referring physician. To reduce the impact of second primary cancer on mortality after ER, we excluded patients with recently diagnosed second primary cancer (other than in situ carcinoma of stomach and colon). Ninety patients without second primary cancer diagnosed within 1 year before ER were defined as subgroup A.

Statistical Analysis

Cumulative survival curves were determined with the Kaplan-Meier method. The significance of differences in survival were assessed by the log-rank test. Cancer patients usually have excess risk in SMR from unity because a certain proportion of cancer patients will die from the underlying cancer, in addition to the expected mortality calculated from the general population. SMR of patients with esophageal cancer is thought to be high because most patients die from esophageal or second primary cancer. Because ER is performed as a curative operation for early esophageal cancer the procedure can be regarded as successful when the SMR is near unity. Therefore, we calculated SMR for the assessment of ER as a treatment for esophageal cancer.

For the calculation of SMR the observed number of deaths was compared with the expected number, which was calculated by applying sex, 5-year age, 5-year calendar time, and cause-specific mortality rates for the general population in the Osaka Prefecture, as prepared by the Statistics and Information Department, Japan Ministry of Health and Welfare.[15] The SMR was expressed by dividing the observed number of deaths in the study group by the expected number of deaths calculated from the general population. The standard error and 95% confidence interval (CI) of SMR were estimated by assuming a Poisson distribution and differences in mortality between the study cohort and the general population were considered significant if the CI did not include unity. Data analysis was performed with SAS/PC statistical package (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics at Entry

A flow chart of patient recruitment is shown in Figure 1. From January 1995 to December 2004, 1049 patients with esophageal cancer visited our hospital. Among these, 159 patients received ER as their first treatment. In all, 110 of 159 patients who received ER fulfilled our inclusion criteria. The characteristics of the study population and lesions are shown in Table 1. The breakdown of second primary cancer was as follows: 14 hypopharyngeal, 3 mesopharyngeal, 11 laryngeal, 5 tongue, 13 gastric, 4 colon, and 3 lung cancers.

ER

A total of 138 ERs were carried out in 110 patients. Detailed data of ER are presented in Table 1. ER was performed in accordance with our inclusion criteria, except for 4 large lesions (>50 mm diameter) that were treated upon the patients' request. Complete local remission was obtained in all lesions after 1 session of ER. The only major complication observed during ER was 1 case of perforation.

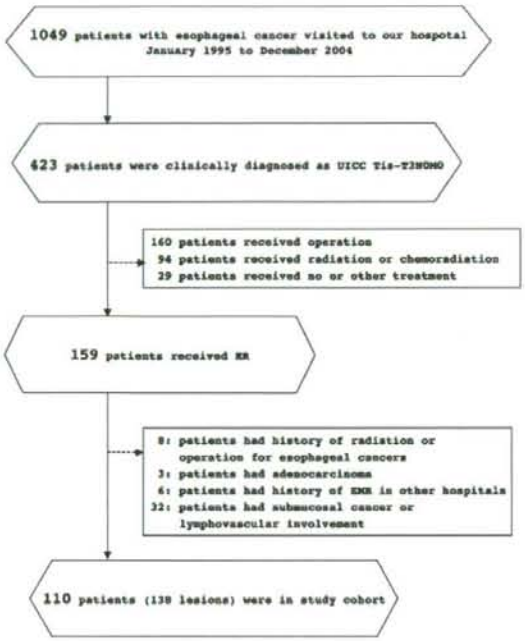

FIGURE 1. Flow chart of patient recruitment.

Follow-up Data

Vital status was unknown in 2 patients who were lost to follow-up at 3 and 16 months after ER. A total of 108 patients (98.2%) were completely followed up, with a mean observation period of 4.7 (range, 0.4–11.3) years. Detailed data of local recurrence, metachronous esophageal cancer, and second primary cancer are shown in Table 2.

Local recurrence was observed in 8 lesions in 8 patients. Seven lesions were successfully treated by additional ER or endoscopic ablation, whereas 1 lesion could not be cured by 2 additional ERs and was successfully treated with chemoradiation.

Eighteen metachronous esophageal cancers in 12 patients were found after ER. Sixteen lesions in 10 patients were treated by ER or endoscopic ablation therapy, and 1 patient was treated by surgical resection. In the other patient, submucosal invasive cancer developed in the cervical esophagus with cervical lymph node metastasis 14 months after ER. The lesions could not be cured by chemoradiation and he died of esophageal cancer 40 months after ER.

Fifteen second primary cancers in 15 patients were found after ER. Ten lesions received curative treatment: surgery for 4 oropharyngeal cancers and 1 lung cancer; radiotherapy for 3 oropharyngeal cancers; and ER for 2 stomach cancers. Two hepatocellular carcinomas developed in 2 patients with cirrhosis and were treated by radiofrequency ablation therapy and transcatheter arterial embolization therapy, respectively. Three patients died of gallbladder cancer, cholangiocarcinoma, and hepatocellular carcinoma, respectively.

TABLE 1
Characteristics of Patients and Lesions at Entry

Characteristics of patients	Total n = 110	Subgroup A n = 90
Mean age, y, (range)	65 (44–84)	65 (44–84)
Male/female	87/23	68/22
Patients with a history of second primary cancer	49	29
Patients with second primary cancer diagnosed within 1 year before ER	20	0
Characteristics of lesions		
No. of lesions	138	111
Mean tumor size, mm (range)	13 (3–70)	13 (3–60)
In situ cancer/invasive cancer	82/56	66/45
Poorly differentiated squamous cell carcinoma	1	1
Method of ER		
EMR/ESD	124/14	100/11
Resection		
En bloc/piecemeal	89/49	72/39

ER indicates endoscopic resection; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection.

We identified 22 deaths during follow-up (Table 3). Death from malignant tumor was identified in 16 (73%) of these. The origin of the malignant tumors was 6 oropharyngeal, 3 lung, 3 liver, 1 laryngeal, 1 esophageal, 1 rectal, and 1 gallbladder. Other causes of death were cerebrovascular disease in 2 patients, heart disease in 2, and cirrhosis in 2 patients (Table 3). In the analysis of mortality data, cerebrovascular disease and heart disease were classified into circulatory disease (International Classification of Diseases). There was no significant difference in survival between lesions of different sizes ($\leq$20 mm vs >20 mm) or depth of invasion (in situ vs invasive lesion).

Survival Curves

Kaplan-Meier curves of all patients and subgroup A were constructed (Fig. 2). The cumulative 5-year survival rate (mean observation period) of all patients and subgroup A was 79.5% (4.7 years) and 86.6% (5.1 years), respectively.

SMR

Differences in mortality in patients from that of the general population were further assessed by calculating SMR. Overall mortality (SMR, 1.68; 95% CI, 1.05–2.55) for patients treated by ER was significantly higher than that of the general population (Table 3).