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c-*myc* mRNA Overexpression is Associated with Lymph Node Metastasis in Colorectal Cancer

K. Sato, M. Miyahara, T. Saito and M. Kobayashi

To elucidate the relationship between oncogene overexpression and malignant potential of colorectal cancer, the expression of three genes, c-*myc*, c-*fos* and c-Ki-*ras* was examined. Northern blot hybridisation was performed on fresh tissues excised from 35 patients with primary colorectal cancer. Overexpression of c-*myc* mRNA was evident in 25 of the patients (71%). Tumours with c-*myc* overexpression had a significantly higher frequency of lymph node metastasis than did those without (68 versus 20%, $P < 0.05$). The survival rate tended to be poorer ($P = 0.06$) in patients with c-*myc* overexpression (79% for 3 years) than in those without it (100%). We found no significant relationship between overexpression of the other two genes and pathological features. These findings suggest that c-*myc* overexpression is one parameter which can be used to determine the malignant potential of colorectal cancer.

Key words: colorectal cancer, c-*myc* mRNA overexpression, northern blot analysis, clinico-pathological findings, prognosis

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INTRODUCTION

PROGNOSTIC FACTORS in case of surgical resection of colorectal cancer are the presence or absence of metastasis [1, 2], depth of tumour invasion and venous and lymphatic invasion [3]. Recent molecular biological studies suggested that estimation of oncogene overexpression may be useful in predicting the prognosis [4, 5].

Certain cellular oncogenes seem to be associated with cellular proliferation, carcinogenesis and tumour progression [6]. Extensive studies on the expression of the oncogenes were conducted

using various tumour cell lines as well as solid tumours [7, 8]. The accumulated data suggested associations between oncogene expression and tumours, including Burkitt lymphoma [9], neuroblastoma [10], retinoblastoma [11], small cell cancer of the lung [12] and breast cancer [13]. In colorectal cancer, c-*myc*, c-Ki-*ras*, c-Ha-*ras* and c-*fos* genes were overexpressed [8], but the associations with malignant potential of the cancer remain controversial [4,14–16]. We examined the relationship between these oncogene overexpressions and clinicopathological features in patients with colorectal cancer.

MATERIALS AND METHODS

Patients

35 Japanese patients with non-hereditary colorectal cancer (19 colon and 16 rectal) were treated in our clinic. The average age was 65.9 ± 9.2 years (range 42–89), 18 patients were male and 17 female. All patients underwent resection of the primary lesion with lymphadenectomy (19 colectomy, 11 anterior resection and 5 amputation of the rectum); 31 underwent curative resection and the remaining 4 with liver metastasis were treated with non-curative resection. For patients with liver metastasis, hepatectomy (2 patients) or hepatic arterial cannulation (2 patients) was added. Oral 1-(2-tetrahydrofuryl)-5-fluorouracil (tegafur) was prescribed postoperatively for all patients. Intra-arterial cisplatin and intravenous 5-fluorouracil were given to the patients with hepatic arterial cannulation.

Resected specimens were examined histopathologically and clinicopathologic evaluations were determined essentially according to rules established by the Japanese Research Society for Cancer of Colon and Rectum [17]; Dukes' classification [18] was also used.

mRNA isolation

Carcinoma and corresponding normal mucosa were taken from resected specimens immediately after excision and stored in liquid nitrogen until use.

Total RNA was prepared from tissues using the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method [19]. The tissues were homogenised in solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol] and the homogenate was added to 2 M sodium acetate (pH 4.0), phenol and chloroform-isoamyl alcohol. Samples were centrifuged at 10000 *g* for 20 min at 4°C. The aqueous phase was transferred to a fresh tube and precipitated with an equal volume of isopropanol at −80°C for 1 h. After centrifugation for 10 min at 4°C, total RNA pellet was extracted and was heated at 65°C for 5 min, then rapidly cooled and applied to a column containing 1 ml of oligo(dT)-cellulose equilibrated with Tris-EDTA solution (TE) containing 0.5 M sodium chloride. After washing of the column with the same buffer, the poly(A)$^+$ RNA was eluted with TE.

Northern blot hybridisation

One microgram of mRNA was separated by electrophoresis in a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The filters were hybridised in solution containing 50% formamide, five times saline sodium citrate (SSC), five times Denhardt's solution (0.1% polyvinyl-pyrrolidone, 0.1% Ficoll and 0.1% bovine serum albumin), 1% sodium dodecyl sulphate (SDS), 5% dextyran sulphate and 100 g/ml salmon sperm DNA at 42°C with ^{32}P-labelled probe, using the random primer method. The probe DNAs (c-*myc*, c-*fos* and c-Ki-*ras*) were provided by the Japanese Cancer Research Bank (JCRB). The c-*myc* gene fragments used in hybridisation were the 420 base pair Pst I exon 2 fragments of human [20]. After hybridisation, the filter was washed with 2 × SSC/0.1% SDS at room temperature and then 0.1 × SSC/0.1% at 65°C. The hybridised filters were exposed to Kodak XAR-5 film at −80°C for 1–5 days.

Statistics

Correlations between overexpression of genes and clinicopathological features were examined using Student's unpaired *t*-test and the χ^2 test. Survival rate was estimated on the basis of the Kaplan–Meier method, and the generalised Wilcoxon test was used for analysis. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Expression of oncogenes

The level of expression of c-*myc*, c-Ki-*ras* and c-*fos* was examined in 35 patients with non-hereditary colorectal carcinoma. The results of northern blot hybridisation showed c-*myc* mRNA transcripts which were 2.7 kb long in the tumour tissues and in the normal mucosa (Figure 1). Expression of β-actin was evaluated as a positive control and β-actin transcript, 2.0 kb long, was similar in all cases. All bands obtained by autoradiography have been measured by a densitometer (Dual wavelength flying scanner CS-9000, Shimadzu, Japan) for objective judgement. The band amount of oncogenes was divided by the band amount of each β-actin, thereby adjusting for the loading amount and a tumour specimen with over four times the content of the normal mucosa has been judged as "positive". Overexpression

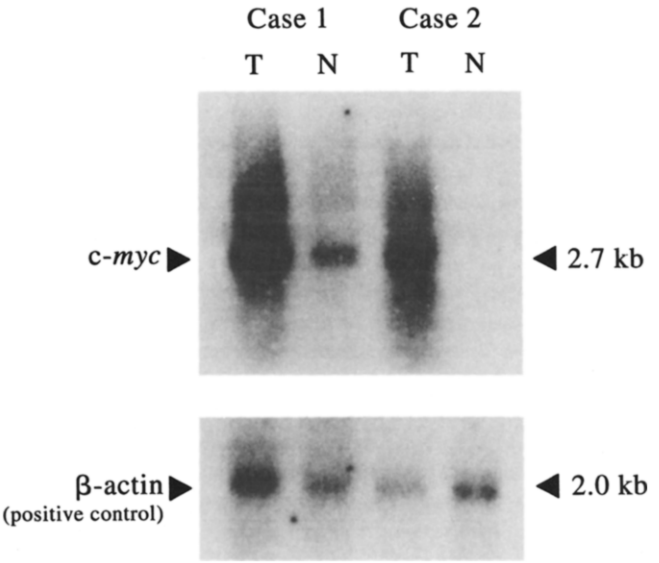

Figure 1. Northern blot analysis of c-*myc* mRNA in two human colorectal tumours (T) and their corresponding normal mucosa (N). c-*myc* RNA ratio T/N was 4.5 in case 1 and 16.8 in case 2. This ratio indicates the fold increase in levels of c-*myc* RNA when a given tumour sample was compared with the normal mucosa adjusted each β-actin level, based on densitometric scanning analysis of the northern blots.

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Table 1. Incidence of overexpression of three oncogenes in colorectal cancer

	Overexpression (%)	
	Positive	Negative
c-*myc*	25 (71)	10 (29)
c-*fos*	16 (46)	19 (54)
c-Ki-*ras*	18 (51)	17 (49)

of the c-*myc* gene was detected in 25 of 35 samples (71%) of the tumour tissues (Table 1). Overexpression of the c-*fos* gene and c-Ki-*ras* was observed in 16 and 18 samples (46 and 51%), respectively.

Expression and clinicopathological findings

Differences between the c-*myc* overexpression positive group and negative group with regard to age (64.6 ± 11.1 versus 69.2 ± 7.9 years) and sex (10 males and 15 females versus eight males and two females) were not significant. Pathological findings of the patients were compared between c-*myc* overexpression positive group and negative group (Table 2). Moderate to poor differentiation tended to be associated with c-*myc* overexpression although there were no statistically significant differences. There was no significant difference between positive and negative expression of the c-*myc* gene in the depth of penetration.

*Table 2. Relationship between c-*myc *overexpression and pathological findings*

	Overexpression (%)	
	Positive	Negative
Differentiation		
Well	5 (20)	5 (50)
Moderately	18 (72)	5 (50)
Poorly	2 (8)	0 (0)
Depth of penetration		
< pm	5 (20)	5 (50)
ss,a1	11 (44)	3 (30)
>s,a2	9 (36)	2 (20)
Lymphatic vessel invasion		
Positive	20 (80)	3 (30)*
Negative	5 (20)	7 (70)
Vessel invasion		
Positive	8 (32)	1 (10)
Negative	17 (68)	9 (90)
Lymph node metastasis		
Positive	17 (68)	2 (20)*
Negative	8 (36)	8 (80)
Liver metastasis		
Positive	4 (16)	0 (0)
Negative	21 (84)	10 (100)
Dukes' classification		
A,B	8 (32)	8 (80)*
C,D	17 (68)	2 (20)

pm; cancer is confined to within the proper muscular layer. ss,a1; cancer invades serosa or adventitia, to some extent. s,a2; cancer invades serosa or adventitia, extensively. *Significant difference ($P < 0.05$).

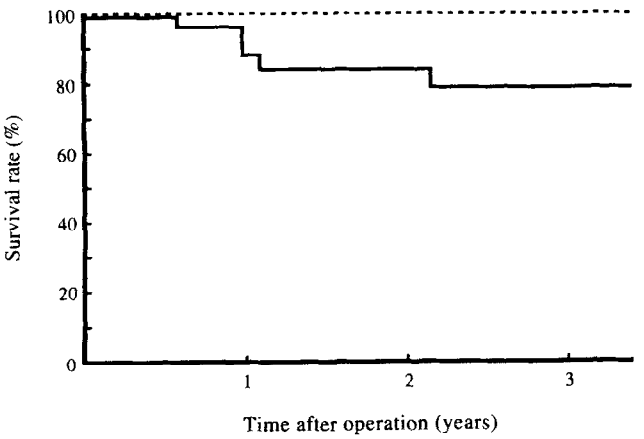

Figure 2. Survival curves of patients with (n = 25,—) or without (n = 10, · · ·) overexpression of c-*myc* gene (P = 0.06).

Lymphatic invasion was present in 20 of the 25 cases (80%) with c-*myc* overexpression and in 3 of 10 (30%) without, a statistically significant difference ($P < 0.05$). No obvious relationship was noted between the c-*myc* expression and venous invasion.

There were lymph node metastases in 68% of those in the c-*myc* expression positive group but in only 20% of those in the negative group, the difference being statistically significant ($P < 0.05$). There was no significant difference in liver metastasis between the two groups although all patients with liver metastasis between the two groups although all patients with liver metastasis showed overexpression of c-*myc*.

There was a significant difference ($P < 0.05$) between the two groups in Dukes' classification. In the case of c-*fos* and c-Ki-*ras* genes, no relationship was noted between the overexpression of genes and clinicopathological features (data not shown).

Survival rate and disease-free survival

Figure 2 shows overall survival curves of the groups, with or without the overexpression of c-*myc*. The 3-year survival rates of the c-*myc* overexpression positive and negative groups were 79.0 and 100%, respectively. Despite a short observation period, the c-*myc* overexpression positive group showed a tendency towards a poorer prognosis as compared with negative groups, albeit statistically non-significant (P = 0.06). Those who died were Dukes' C, 2 cases, and Dukes' D, 3 cases. Figure 3 shows

Figure 3. Disease-free curves of the patients with curative resection within (n = 21,—) or without (n = 10, · · ·) overexpression of c-*myc* gene (P = 0.06).

Table 3. Recurrent cases

Cases	Age	Sex	Primary lesion	c-*myc* expression	Disease-free interval (months)	Recurrent lesion
1	74	F	Rectum	Positive	25	Skin
2	69	M	Rectum	Positive	17	Liver
3	52	M	Ascending	Positive	16	Liver
4	59	M	Rectum	Negative	30	Local
5	82	F	Ascending	Positive	11	Lung
6	80	F	Sigmoid	Positive	14	Liver
7	69	M	Rectum	Positive	18	Lung

F, female; M, male.

disease-free survival curves of the two groups for the cases with curative resection. The 3-year disease-free survival rates were 72.0% (positive group) and 83.0% (negative group). The positive group showed a tendency towards a poorer prognosis as compared with the negative group, although the difference was non-significant ($P = 0.06$). All recurrent cases are shown in Table 3. Liver metastasis occurred in 3 cases and lung metastasis in 2 cases. Skin metastasis and local recurrence occurred in 1 case each.

DISCUSSION

To design effective therapy for patients with a malignant tumour, the biological behaviour of the tumour has to be defined. In colorectal cancer, factors such as the presence or absence of tumour invasion, depth of tumour invasion and venous and lymphatic invasion represent the grade of malignant potential and the prognosis can be estimated. During the past decade, several studies suggested that the biological behaviour of a malignant tumour is reflected by oncogene expression.

Expression of the c-*myc* gene increases when quiescent cells are stimulated to proliferate [21] by mitogen, and it is invariant throughout the cell cycle in proliferating cells [22, 23]. Furthermore, the expression of the c-*myc* gene is downregulated when immature haematopoietic cells [24] or teratocarcinoma stem cells [25] are induced to differentiate into non-proliferative cell types. Thus, the c-*myc* gene plays an important role in cellular proliferation.

Sikora *et al.* reported expression of the c-*myc* gene in resected human colonic cancers and considered how it may relate to prognosis [4]. Rowley *et al.* noted that low levels of c-*myc* p62 protein observed with flow cytometry correlated with improved survival rates and that this was a better prognostic index than DNA ploidy in patients with colorectal cancer [5]. In contrast, Erisman *et al.* found no association of c-*myc* expression with survival [14]. In other studies, expression of the c-*myc* gene and c-*myc* p62 protein did not correlate with depth of tumour invasion and stage grouping [15, 26]. Thus, the controversy remains unresolved [16, 27, 28]. In our study, northern blot analysis was performed to visualise the expression of mRNA. In most studies by other workers, total RNA was used. However here, poly(A)$^+$ RNA samples purified oligo(dT)cellulose column were used to prepare mature mRNA to exclude non-specific hybridisation and to obtain a higher precision result.

The novel observation of the present study was that overexpression of the c-*myc* gene related to lymphatic invasion, lymph node metastasis and Dukes' classification, in a statistically significant manner. Therefore, colorectal cancer with c-*myc* overexpression suggests a malignancy associated with lymph node metastasis. Furthermore, our study showed that patients with c-*myc* overexpression tended to have a poorer prognosis. The determination of c-*myc* expression may contribute to prediction of biological tumour behaviour in colorectal carcinomas, but longer observation periods and larger numbers of cases are necessary before any conclusion can be reached.

The c-*fos* gene is involved in cellular differentiation and growth regulation and c-Ki-*ras* gene alternation is associated with the early period of carcinogenesis. Overexpression of c-*fos* and c-Ki-*ras* genes in tumour tissues was noted with a high incidence, but did not correlate with any clinicopathological features in our study. Of the three gene expressions examined, only that of the c-*myc* was significant.

These findings suggest that c-*myc* overexpression is one parameter which can be used to determine the malignant potential of colorectal cancer.

Detection of c-*myc* overexpression by northern blot hybridisation is one useful indicator for clinical evaluation of a malignancy in the colorectal region, especially on lymph node metastasis.

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Influence of Dexniguldipine-HCl on Rhodamine-123 Accumulation in a Multidrug-resistant Leukaemia Cell Line: Comparison With Other Chemosensitisers

R. Boer, S. Haas and A. Schödl

In the clinical therapy of cancer, resistance to many cytostatic drugs is a major cause of treatment failure. Among other mechanisms, the expression and pumping activity of P-glycoprotein (PGP) in the membrane of resistant cancer cells is responsible for the reduced uptake of cytostatics. The blockade or inhibition of PGP activity by chemosensitisers seems to be a tenable way to restore sensitivity to antineoplastic drugs and therapeutic efficacy. In the present work the influence of the new chemosensitiser dexniguldipine on rhodamine-123 accumulation in multidrug-resistant leukaemia cells was investigated. Dexniguldipine increases cellular rhodamine-123 accumulation dose-dependently. pEC_{50} values (−log concentration of drug showing a half maximal effect) in accumulation studies are dependent on pH of the test system and are in the range of 6.5 (pH 7.2) to 7.2 (pH 8.0) for dexniguldipine. In comparison with other chemosensitisers such as SDZ PSC 833, cyclosporin A, verapamil, dipyridamole, quinidine and amiodarone, dexniguldipine is the most potent drug in this test system. In addition to equilibrium measurements of rhodamine-123 accumulation, efflux of rhodamine-123 was analysed in the absence and presence of chemosensitisers. A clear dose-dependency was seen and, moreover, a dramatic decrease in efflux rates was achieved in the presence of chemosensitisers. The described system can be used to investigate PGP-mediated drug transport on a pharmacological and biochemical basis.

Key words: rhodamine-123 uptake, MDR cells, dexniguldipine, SDZ PSC 833, chemosensitiser, resistance reversal, rhodamine-123 efflux

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INTRODUCTION

THE SUCCESSFUL treatment of cancer with cytostatic drugs is often impaired by the development of resistance to these substances. There are multiple mechanisms which can lead to resistant cancer cells. The best studied and probably most frequent mechanism is the appearance and overexpression of P-glycoprotein (PGP), whose pumping activity reduces the intracellular drug concentrations below the effective levels [1, 2]. PGP shows a rather non-specific substrate specificity in removing drugs of different chemical classes with different