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Multidisciplinary approach to a case of Lynch syndrome with colorectal, ovarian, and metastatic liver carcinomas

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Abstract Lynch syndrome is an autosomal dominant disorder with an estimated prevalence of 3 % of all colorectal cancers. It is attributed to germline mutations in DNA mismatch repair (MMR) genes, which confer increased susceptibility to cancers of the colorectum, endometrium, stomach, small intestine, hepatobiliary system, kidney, urinary bladder, brain, and ovary. We report a thought-provoking Lynch syndrome case with a family history and simultaneous tumors in the colon, pelvis, and liver. These findings made diagnosis and treatment complicated. However, the multidisciplinary approaches followed by a medical oncologist, gynecologist, surgeon, radiologist, and pathologist led to a favorable outcome. This patient had two primary cancers of the colon and ovary, and systemic metastases of colon cancer. The loss of MSH6 protein expression was proven by immunohistochemical examination, but the germline *MSH6* mutation was not detected by DNA sequence analysis. Regarding this discrepancy, some possibilities, e.g., genomic rearrangements and epigenetic modifications, which can be missed by conventional sequence analysis, were considered. Theoretically, Lynch syndrome cases with MSH6 impairment exhibit late onset and low penetrance compared to other major cases with *MLH1* or *MSH6* mutations. Irinotecan hydrochloride (CPT-11) has favorable effects on MMR-deficient tumor cells with high microsatellite instability, although its clinical benefit remains controversial. In this case, the first-line chemotherapy bevacizumab + FOLFIRI regimen has been effective for over a year in the partial response state. We discuss the diagnostic, therapeutic, pathological, and molecular biological characteristics of this intriguing case, indicating the importance of family history, histological assessment, and molecular biological etiology in Lynch syndrome cases presenting a complicated phenotype.

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Keywords Lynch syndrome · Family history · *MSH6* · Irinotecan (CPT-11) · Multiple cancers

Case presentation

Dr. Ishioka (medical oncologist, chairperson of the conference): Good evening, everyone. Today, we would like to discuss a thought-provoking Lynch syndrome case. (*A brief summary of the case is given in the Abstract*). Dr. Shiono, please begin the case presentation.

Dr. Shiono (medical oncologist, physician in charge of this case): A 51-year-old woman, diagnosed with advanced colon cancer with multiple liver metastases, was referred to our outpatient department by her primary practitioner for systemic chemotherapy.

The patient had been well until 3 weeks before a visit to her doctor for right upper quadrant pain. Abdominal ultrasound revealed multiple masses in the liver. Subsequent computed tomography (CT) revealed metastases from an unknown origin (Fig. 1a), and a pelvic mass was considered as a right ovarian mucinous cystadenoma (Fig. 1b). While esophagogastroduodenoscopy (EGD) detected no lesions, colonoscopy disclosed a type 2 tumor in the sigmoid colon, which was histologically diagnosed as a poorly differentiated adenocarcinoma (Fig. 1c). There was nothing in particular to declare in the patient's past medical and social histories. She had never been married or pregnant, and was post-menopausal. Her family history revealed a background of Lynch syndrome. Her father had been diagnosed with colorectal cancer at the age of 42, and her two paternal uncles, aunt, and grandmother also had colorectal cancer (Fig. 2). This patient was diagnosed with Lynch syndrome by fulfilling the Amsterdam criteria II [1]. No apparent abnormalities were observed on physical examination. Laboratory data showed anemia (Hb 8.9 g/dl) and elevated CEA (1480 ng/ml) and CA19-9 (2418 U/ml) levels.

Differential diagnosis

To identify potential genes for Lynch syndrome, we submitted a colon cancer biopsy specimen for immunohistochemical (IHC) examination of the DNA mismatch repair (MMR) gene products, i.e., MLH1, MSH2, MSH6, and PMS2. Because Lynch syndrome was diagnosed, we could not completely rule out the possibility of ovarian cancer. An effective chemotherapy regimen should be selected based on the origin of the liver metastases. Hence, we consulted a radiologist and gynecologist for differential diagnoses of the pelvic tumor.

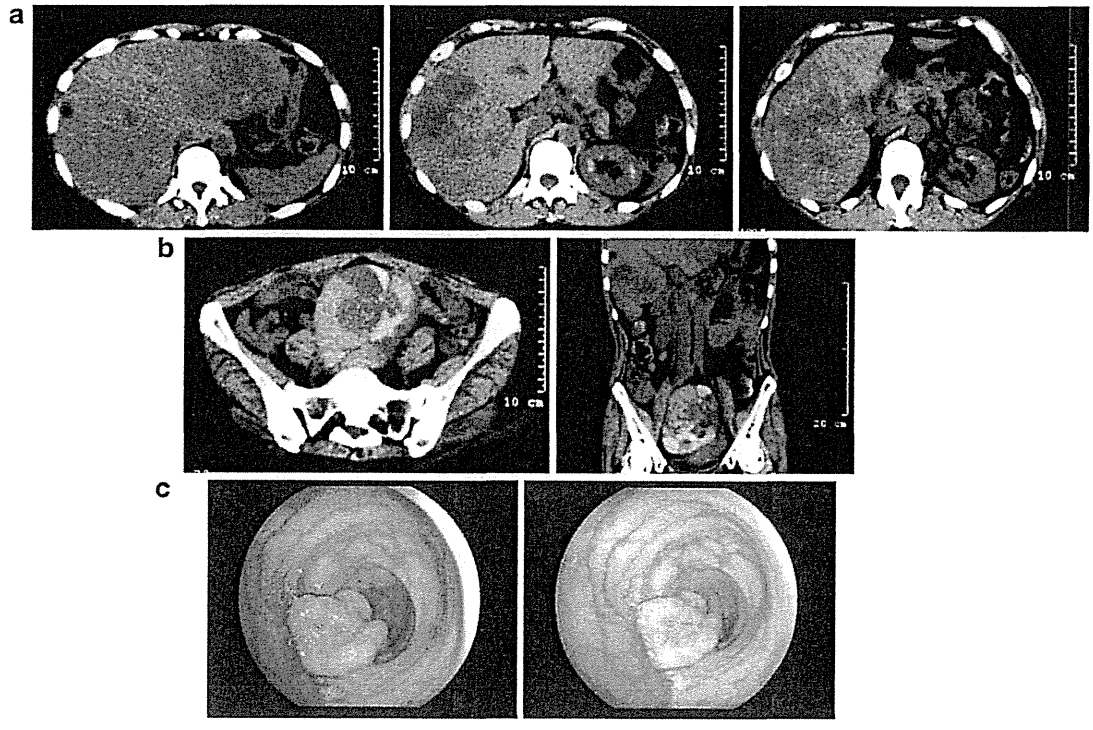


Fig. 1 CT images and colonoscopy findings at onset. **a** Axial images of the liver. Multiple low-density areas suggest metastases. **b** Axial and coronal images of the pelvic tumor. Various densities ranging from low to high, suggesting various liquid and solid components, are seen. **c** Captured images during colonoscopy. A massive type 2 tumor is seen in the sigmoid colon

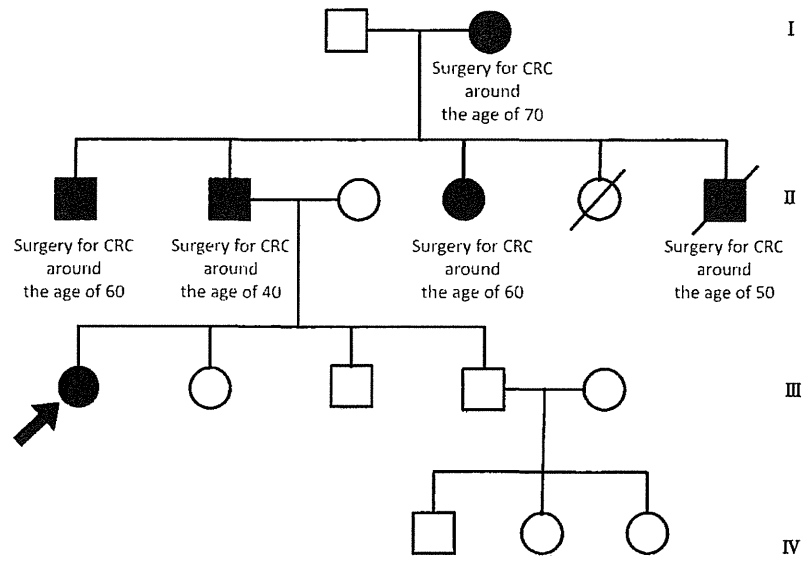


Fig. 2 Family tree. Five people were affected with colorectal cancer in the first degree relatives of the patient's father among three generations. Lynch syndrome was diagnosed, which fully met the diagnostic criteria of Amsterdam II. *Squares* and *circles* indicate male and female, respectively. *Arrow* indicates the patient. *Filling with black* indicates affected person with trait. *Diagonal line* indicates deceased relatives. *CRC* colorectal cancer

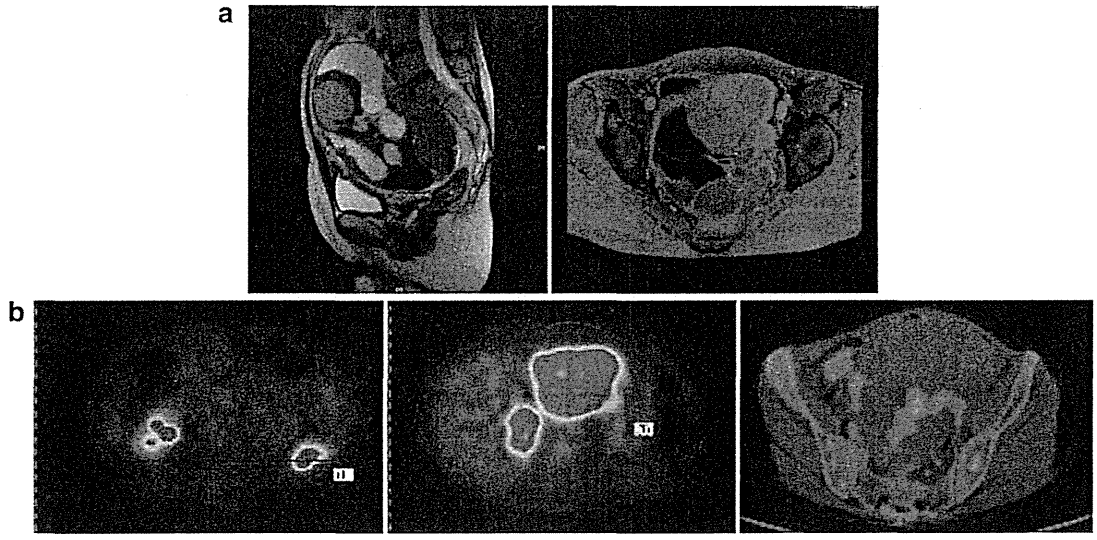


Fig. 3 PET–CT and MRI images 1 month after onset. **a** MRI images of the pelvic tumor. Mixtures of diverse intensities ranging from low to high, suggesting a variety of liquid and solid components, are seen. There seems to be a hemorrhage and mucus in it. Strong enhancement is seen in the cyst wall. A multilocular cystic ovary including a metastatic lesion of cyst walls with contrast enhancement was possibly suggested. **b** Axial PET–CT fusion images of the colon, liver, and ovary. SUV_{max} values of 9.0 in the colon, 7–10.0 in the liver, and 4.0 in the ovary were detected

Dr. Takase (*radiologist*): The pelvic mass was a multilocular cystic tumor, which showed various signals and densities on magnetic resonance imaging (MRI) and CT, respectively, presumably from a hemorrhage and mucus (Fig. 3a, left). Strong enhancement was observed in the cyst wall on MRI, although the solid part was minimal (Fig. 3a, right). A positron emission tomography/CT (PET–CT) image showed various maximum standardized uptake values showing malignancy (SUV_{max} 9.0 in the colon, 7.0–10.0 in the liver, and 4.0 in the ovary) (Fig. 3b). It is difficult to determine whether an ovarian tumor is primary or metastatic when another definitive tumor is apparent [2, 3]. Most metastatic ovarian tumors show solid and cystic components, but a cyst is not evidence of primary ovarian cancer. Unlike metastatic tumors of other organs, it is common for metastatic ovarian tumors to contain cysts even if the primary site solely consists of a solid mass. When an ovarian cystic tumor and primary cancer are observed simultaneously, we first consider the possibility of a metastatic ovarian tumor. However, it was quite difficult to distinguish the masses through imaging. We thought that this might be a multilocular cystic ovary

with metastatic lesions of the cyst walls with contrast enhancement [4].

Dr. Ito (gynecologist): Because few solid parts were present, which is often the case with primary ovarian cancer, in addition to normal CA-125 levels, a borderline tumor was conceivable in this case. However, laparotomy and histopathological assessment were necessary for the definitive diagnosis.

Initial treatment plan and its course

Dr. Shiono: Given the patient's history, we decided to prioritize chemotherapy for colorectal cancer, which had already been diagnosed as malignant. Considering her Lynch syndrome background, we selected an irinotecan hydrochloride (CPT-11)-based bevacizumab + FOLFIRI regimen. After confirming the uridine-5′-diphosphate-glucuronosyltransferase 1A1 (UGT1A1) *6 and *28 status as wild type for CPT-11 use, she was admitted for central venous port implantation for outpatient chemotherapy.

Because we used the biopsy specimens for MMR IHC, we performed colonoscopy to obtain biopsy samples for *KRAS* gene mutation analysis.

Dr. S. Takahashi (medical oncologist, operator of colonoscopy): Compared to the photograph taken by the former endoscopist 2 months earlier, the tumor had grown so rapidly that the lumen was subtotally occluded (Fig. 4a). Taken together with the fact that colon-cleaning preparation required considerable time, stenosis seemed to be severe. After acquiring the biopsy specimen, we performed CT to assess the indication for preemptive surgery for preventing mechanical colonic obstruction by the tumor.

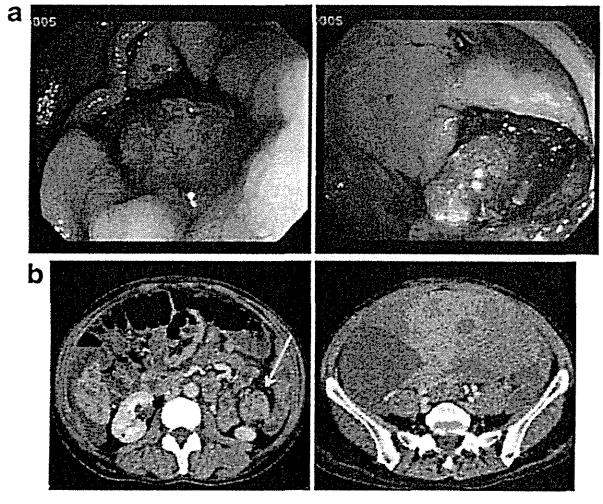


Fig. 4 Colonoscopy and CT images 2 months after onset. **a** Captured images during colonoscopy. Compared with Fig. 1c, the tumor had grown so rapidly that the lumen was subtotally occluded. **b** CT images of the colon and pelvic tumors. *Left panel* Upper colon from the stenosis site at the sigmoid with a massive tumor is enlarged. *Arrow* indicates the lesion. *Right panel* Pelvic tumor is also extremely increased in size compared with that in the former images

Dr. Takase: The upper colon from the stenosis site at the sigmoid with massive tumor was enlarged. Compared with that in the CT images obtained at the former hospital, the ovarian tumor was also extremely enlarged (Fig. 4b).

Dr. Shiono: We consulted a surgeon for palliative surgery, planned elective operation, excluded bevacizumab to avoid interference with postoperative wound healing, and administered FOLFIRI chemotherapy (*l*-LV 275 mg, CPT-11 220 mg, 5-FU bolus i.v. 570 mg, 5-FU c.i.v. 3500 mg) once during the preoperative waiting period.

Preoperative clinical diagnosis

1. Lynch syndrome
2. Colorectal cancer
3. Ovarian tumor, borderline tumor suspected
4. Metastatic liver tumor
5. Subileus due to mechanical obstruction by colorectal cancer

Dr. Ishioka: Please tell us the operative findings, Dr. Miura.

Dr. Miura (surgeon): First, an infant head-sized multilocular and partially villous right ovarian tumor was seen. The left ovary had shrunk. In the abdominal cavity, the disseminated lesion and a small amount of pale yellow ascites were observed at vesicouterine and Douglas pouches, which were considered to be derived from right ovarian cancer. On the other hand, there was a near circumferential 50-mm tumor in the middle portion of the descending colon. However, serous surface invasion was not recognized macroscopically. Multiple metastatic tumors were observed on the bilateral liver lobe, presenting the so-called state of "tumor liver." Because of diffuse intra-abdominal adhesions due to peritonitis carcinomatosa (PC) and definitive prognostic factors such as tumor liver or PC, we performed minimally invasive, palliative, and debulking surgery, i.e., descending colectomy, oophorectomy, and liver biopsy.

Pathological discussion

Dr. Ishioka: Dr. Watanabe, please explain the pathological findings.

Dr. Watanabe (pathologist): A circumferential type 2 tumor (30 × 25 mm) was observed in the descending colon (Fig. 5a). This loupe image illustrates the part of the tumor penetrating the serosa (Fig. 5b). Histologically, a moderately differentiated tubular adenocarcinoma (tub2)

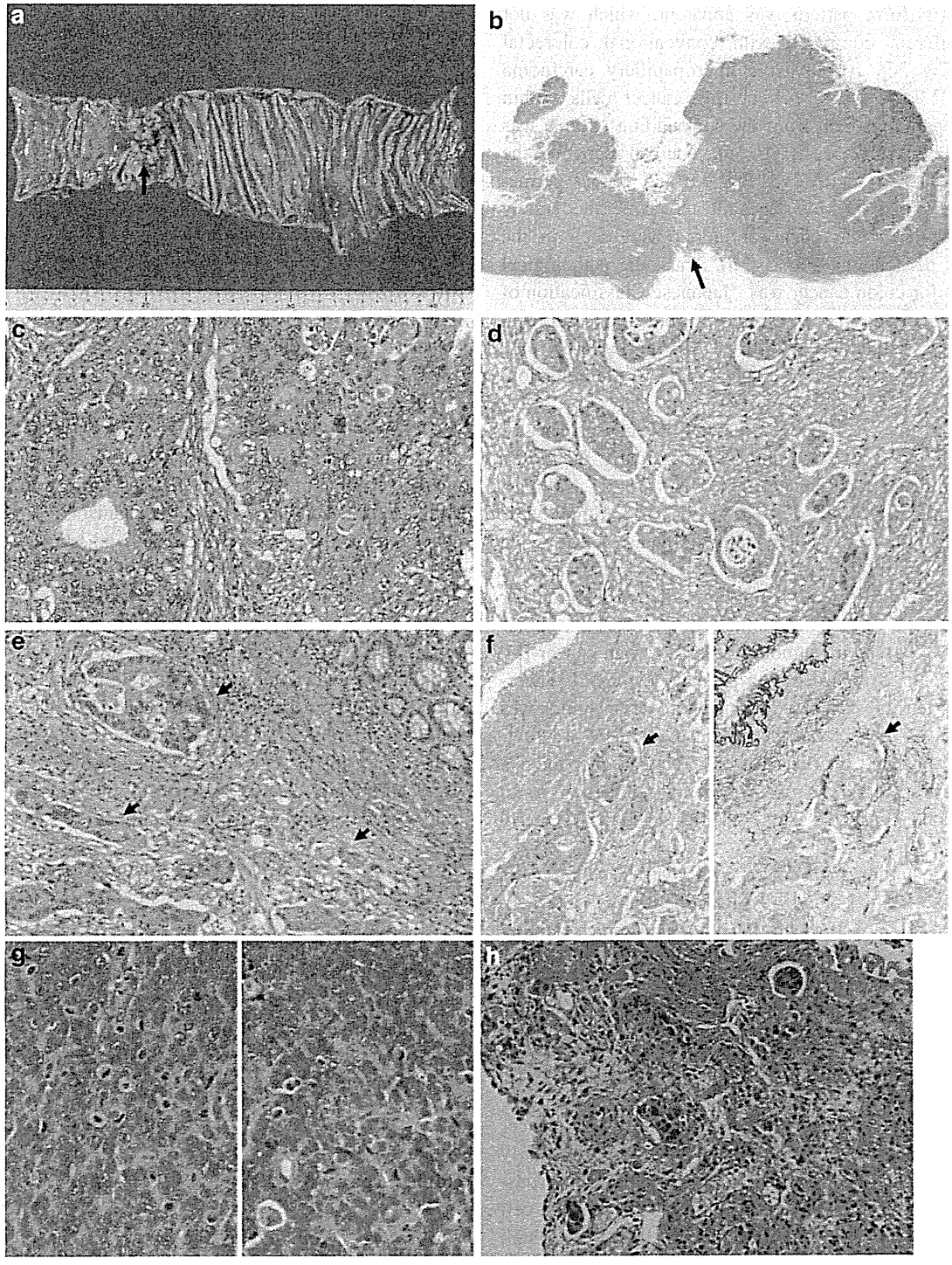


Fig. 5 Pathological findings of the descending colon cancer. **a** Macroscopic view of the resected descending colon with the cancer. A circumferential type 2 tumor (30 × 25 mm) is seen. **b** Loupe view. *Arrow* indicates the part where cancer cells penetrated the serosa. **c** Detailed microscopic view. Moderately differentiated tubular adenocarcinoma (tub2) with a cribriform pattern is apparent. There is not much difference compared with conventional colorectal cancer. **d** An invasive micropapillary carcinoma pattern (IMPC). Nesting cancer cells within spaces separating themselves from the surrounding stroma is seen in the invasive area. **e** Lymphatic involvement. *Arrows* indicate the parts. **f** Venous involvement. *Left panel* H&E stain. *Arrow* indicates the parts. *Right panel* Elastica-Masson stain for veins. *Arrow* indicates the same parts of the *left panel*. Liver (**g**) and peritoneal (**h**) metastases. The histologically similar, moderately differentiated, tubular adenocarcinomas originated from primary colon cancer are seen

with a cribriform pattern was apparent, which was not much different compared with conventional colorectal cancer (Fig. 5c). An invasive micropapillary carcinoma pattern (IMPC), which has nesting cancer cells within spaces separating them from the surrounding stroma, was seen in the invasive area (Fig. 5d). You can recognize the lymphatic involvement (1y3, Fig. 5e) and venous permeation (v2, Fig. 5f). Metastases of the same moderately differentiated tubular adenocarcinoma were seen in the liver and peritoneum (Fig. 5g, h). Thus, the pathological diagnosis for colon cancer was "Japanese Classification of Colorectal Carcinoma: D, type 2, 30 mm, tub2, pSE, int, INFb, ly3, v2, bud(−), pPM0, pDM0, pN1 (1/15), pH1 (grade A), pP1, Cy1, cM0, stage IV; TNM classification: pT4a, pN1a, pM1b, G2, stage IVB."

Although it was difficult to decide between primary or metastatic based on resemblance, we finally diagnosed primary ovarian cancer. The right ovary (180 × 150 mm) consisted of cystic and solid parts (Fig. 6a). A loupe view showed papillary or solid tumor growths beside wide necrotic lesions in the cyst (Fig. 6b). Columnar atypical cells with tubular formation similar to that of colon cancer were also observed (Fig. 6c). An ovarian metastatic tumor can morphologically resemble primary ovarian cancer [5], and the colon is regarded as a primary lesion [6]. Therefore, it is feasible to presume that the ovarian tumor was metastasis of the colon cancer. However, there was evidence of primary ovarian cancer. You can recognize a definitive transitional lesion from benign epithelium to an atypical one (Fig. 6d). This "in situ lesion" is clearly primary.

In IHC studies, both colon and ovarian tumor cells showed CA125(−), CA19-9(+), vimentin(−), CK7(−), CK20(+), CDX2(+), resembling colorectal cancer staining patterns (Fig. 6e, f). Yet, the characteristic difference between them was the staining pattern of PTEN, which supports the likelihood of ovarian serous adenocarcinoma (Table 1). Hence, the ovarian tumor was diagnosed as "serous adenocarcinoma, TNM classification: pT1c, cN0, cM0, G1, FIGO stage IC."

The features of Lynch syndrome-related ovarian cancer are as follows: young onset (mean age 48 years), early stage (FIGO stage I, 47 %), comparatively frequent serous-type histology (endometrioid 35 %, serous 28 %, clear cell 17 %, mucinous 5 %, undifferentiated 15 %), and high attribution rate of *MSH6* deficiency among underlying MMR gene mutations (*MSH2* 49 %, *MSH6* 33 %, *MLH1* 17 %) [7].

Dr. Ishioka: Please describe the MMR IHC results.

Dr. Shimodaira (*medical oncologist*): Whereas MLH1, MSH2, and PMS2 showed nuclear staining patterns indicating intact expression, MSH6 did not (Fig. 7). Thus, *MSH6* must be responsible gene for this case.

Fig. 6 Pathological findings of the right ovarian cancer. **a** Macroscopic view of the resected right ovary with the cancer. It is 180 × 150 mm in size and consisted of cystic and solid parts. **b** Loupe view. Papillary or solid growths of the tumor besides wide necrotic lesions are seen in the cyst. **c** Detailed microscopic view. Some columnar atypical cells with tubular formation similar to that of colon cancer were seen. **d** In situ lesion. There are epithelial cells overlaying the inner surface of the cyst, which shows definitive transitional lesions from benign epithelium to an atypical one. The in situ lesion is evidence of primary ovarian cancer. **e** IHC studies for CK7 (*left upper and right upper panels*) and CK20 (*left lower and right lower panels*). *Left upper and left lower panels* Colon cancer. *Right upper and right lower panels* Ovarian cancer. **f** IHC studies for CDX2 (*left upper and right upper panels*) and CA125 (*left lower and right lower panels*). *Left upper and left lower panels* Colon cancer. *Right upper and right lower panels* Ovarian cancer ▶

Dr. Ishioka: What were the results of sequence analysis?

Dr. Shiono: Despite the above-mentioned IHC results of the colon cancer specimen, we could not detect any pathogenic germline mutations in *MLH1*, *MSH2*, and *MSH6* by direct sequence analyses. The mechanism of that divergence was unclear and will be discussed later.

Moreover, the genetic status of *KRAS* was wild type with regard to inspected codons 12 and 13.

Dr. Miura: That was very interesting. Concerning the differential diagnosis of the pelvic tumor, many organs could be the candidate origin, e.g., colon, bladder, prostate, ovary, and uterus. Lastly, we proposed IHC marker sets as the screening criteria [8]. Although these sets seemed unnecessary in this case because detailed molecular analyses had already been performed, they might be useful in other cases depending on the situation.

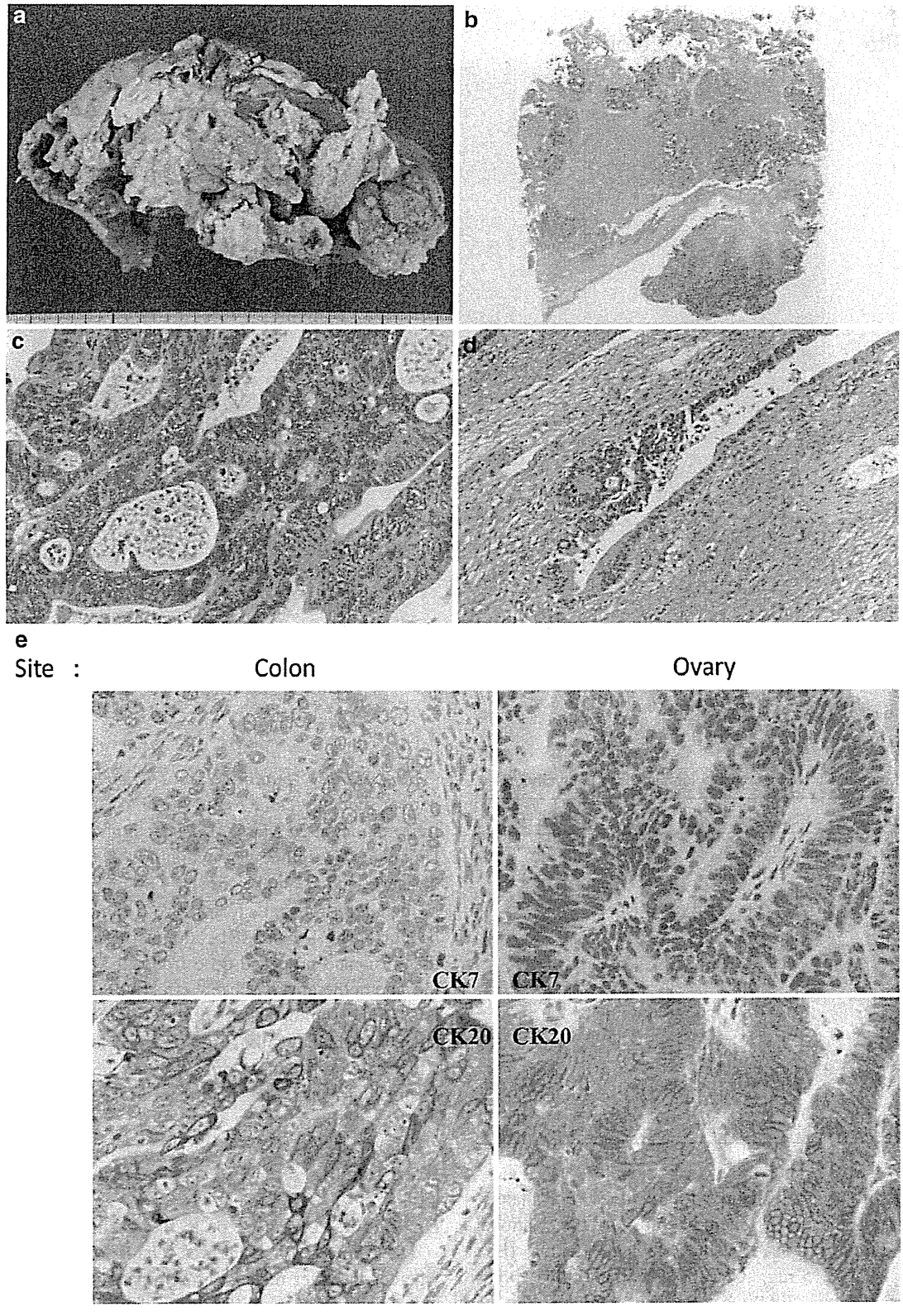
Final diagnosis

1. Lynch syndrome with MSH6 deficiency
2. Descending colon cancer (tub2, pT4a, pN1a, pM1b, stage IVB) with multiple metastases to the lymph nodes, liver, and peritoneum
3. Right ovarian cancer (serous adenocarcinoma, pT1c, cN0, cM0, G1, FIGO stage IC)

Clinical course

Dr. Ishioka: Well, tell us the clinical course after that, please.

Dr. Shiono: The postoperative course was favorable. The first visit day after discharge to restart bevacizumab + FOLFIRI therapy was 11 March 2011. While in the waiting room of the Tohoku University Hospital Cancer Center, the Great East Japan Earthquake occurred. Because she resided in the coastal area, she lost her house in the tsunami. Therefore, she moved to Fukuoka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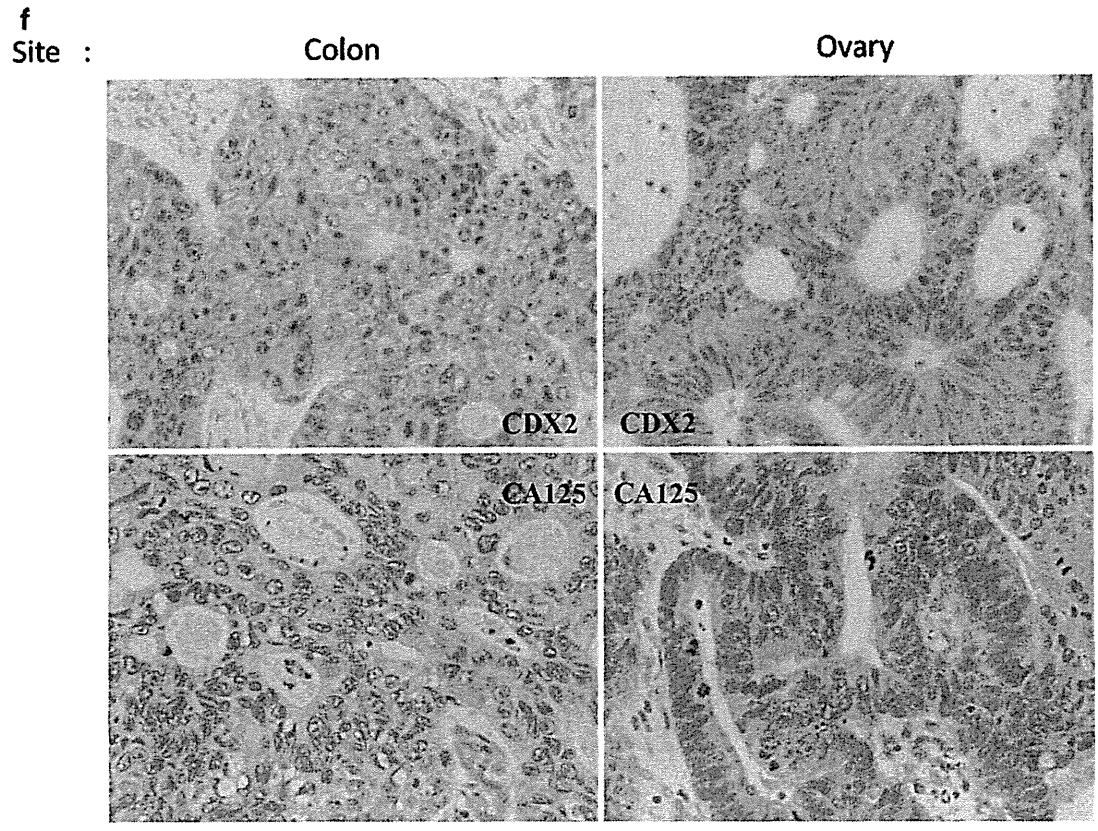

Fig. 6 continued

Table 1 Immunohistochemistry of colon and ovarian carcinomas

	Colon	Ovary
CK7	−	−
CK20	+	+
CDX2	+	+
CA125	−	−
CEA	++	++
ER	−	−
PgR	−	−
p53	+	+
p16	Focal+	Focal+
PTEN	Focal+	Diffuse++
Vimentin	−	−

prefecture on Kyushu Island with relatives. Fortunately, she restarted the same chemotherapy at the National Hospital Organization Kyushu Medical Center (Bev 230 mg, *l*-LV 275 mg, CPT-11 200 mg, 5-FU bolus 560 mg, 5-FU civ. 3000 mg). She has remained in the partial response (PR) state over a year.

Dr. Ishioka: We have a comment from Dr. Takami, who is in charge at Kyushu Medical Center. Please read it for us.

Dr. Shiono (*reading Dr. Takami's comment*): The patient suddenly came to our hospital without any medical information on 22 March. Luckily, a phone line to Tohoku University Hospital was available on that day after the disaster, and I spoke to Dr. Shiono. After receiving a detailed referral form, we immediately initiated bevacizumab + FOLFIRI administration based on the diagnosis and proposed dose from 29 March. Fortunately, the chemotherapy has been effective. The tumor marker levels and tumor sizes of the liver metastases have decreased dramatically (Fig. 8a, b). One year later, she is still receiving benefits from first-line chemotherapy, which is amazing considering her status of severe metastases.

Discussion

Dr. Ishioka: Let's move on to the discussion.

Dr. Shiono: First, it was challenging to determine whether the liver metastases originated from the colon or ovary because of the difficulty in the differential diagnosis of the pelvic tumor. In this case, the clinical response to the regimen was favorable, which was consequently in line with the histopathological assessment obtained via

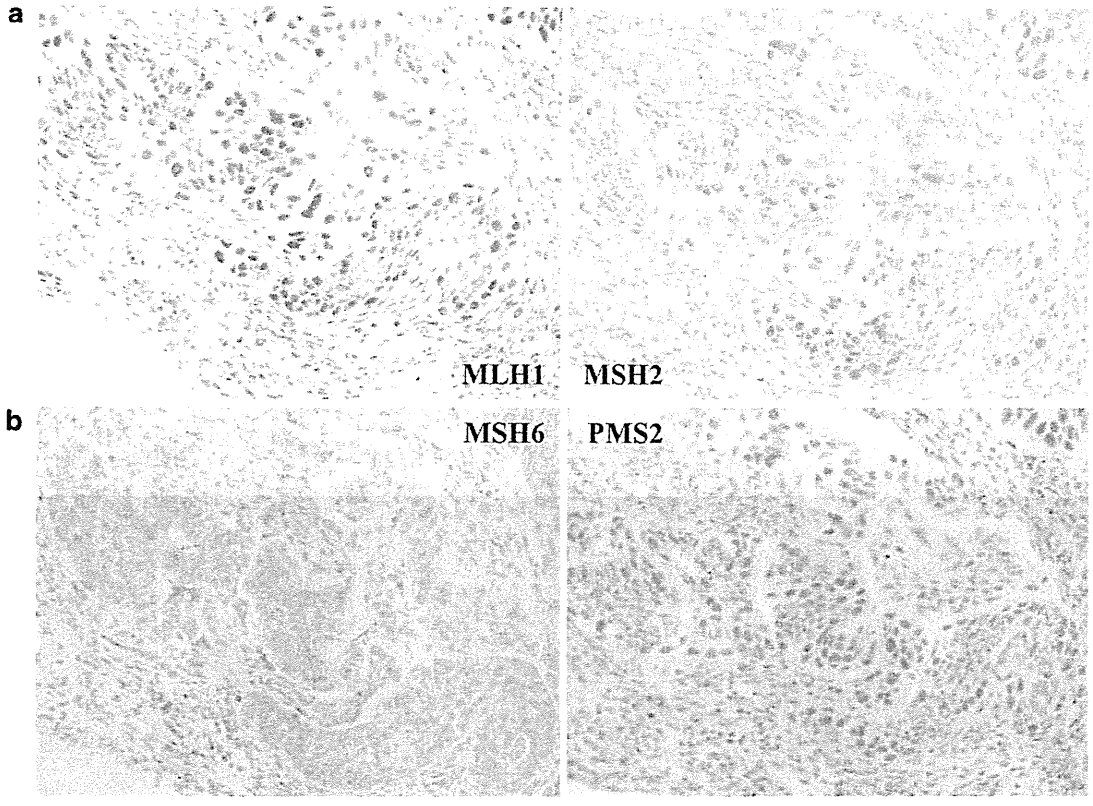

Fig. 7 IHC for MMR proteins using the colon cancer specimen. **a**, **b** IHC studies for MLH1 (*left upper panels*), MSH2 (*right upper panels*), MSH6 (*left lower panels*), and PMS2 (*right lower panels*). Whereas MLH1, MSH2, and PMS2 showed the nuclear staining pattern, MSH6 did not. *Left lower panel* Cytoplasmic weak and blur staining was regarded as non-specific compared to other positive and negative controls

palliative surgery. Thus, it is important to select a suitable regimen in accordance with the histology, if possible.

Dr. Ishioka: What about the practical treatment?

Dr. Shiono: In the literature, CPT-11 is clearly effective on MMR-deficient tumor cells in vitro and favorable in some clinical studies, but its clinical evidence remains controversial [9–17]. Hence, we selected the bevacizumab + FOLFIRI regimen as first-line chemotherapy for the metastatic colorectal cancer. In retrospect, because it has still been effective in the PR state for over a year, the chemotherapy choice seems to be reasonable in this case.

Dr. Ishioka: Please explain the standard first-line chemotherapy for advanced or metastatic colorectal cancer, Dr. Kakudo.

Dr. Kakudo (*medical oncologist*): There are some options. As first line chemotherapy, we choose FOLFIRI or FOLFOX (5-FU/*l*-LV/*l*-OHP) regimens as a combination of cytotoxic agents. Sequential therapy like FOLFIRI or FOLFOX regimen as first line, followed by the alternative regimen as second line, has improved outcome regardless of the order of the regimens [18]. CapeOX, the regimen using the oral prodrug of 5-FU, is another option showing an almost identical outcome compared to FOLFOX [19]. The common adverse events are different: peripheral neuropathy in *l*-OHP and diarrhea in CPT-11. The last choice is whether to add molecular-targeted agents such as bevacizumab (anti-VEGF antibody drug) or cetuximab/panitumumab (anti-EGFR antibody drugs). You should pay attention to contraindications of these monoclonal antibody drugs, e.g., the comorbid severe vascular problems in bevacizumab use. Patients with the *KRAS* gene mutation should be excluded from cetuximab/panitumumab administration. We make an optimal decision depending on the circumstances of each case [20, 21].

Dr. Shiono: In this case, CPT-11 was used as a key drug considering the Lynch syndrome background. CPT-11's effectiveness against cancer cells resulting from a MMR deficiency has been demonstrated in in vitro analyses. Although the entire mechanism remains unclear, it is speculated that CPT-11, an topoisomerase-I inhibitor, exerts its cytotoxicity by generating DNA double-strand breaks (DSBs) in the administered cell. Conversely, MMR-deficient tumor cells have a tendency to accumulate mutations within microsatellite repeats of genes associated

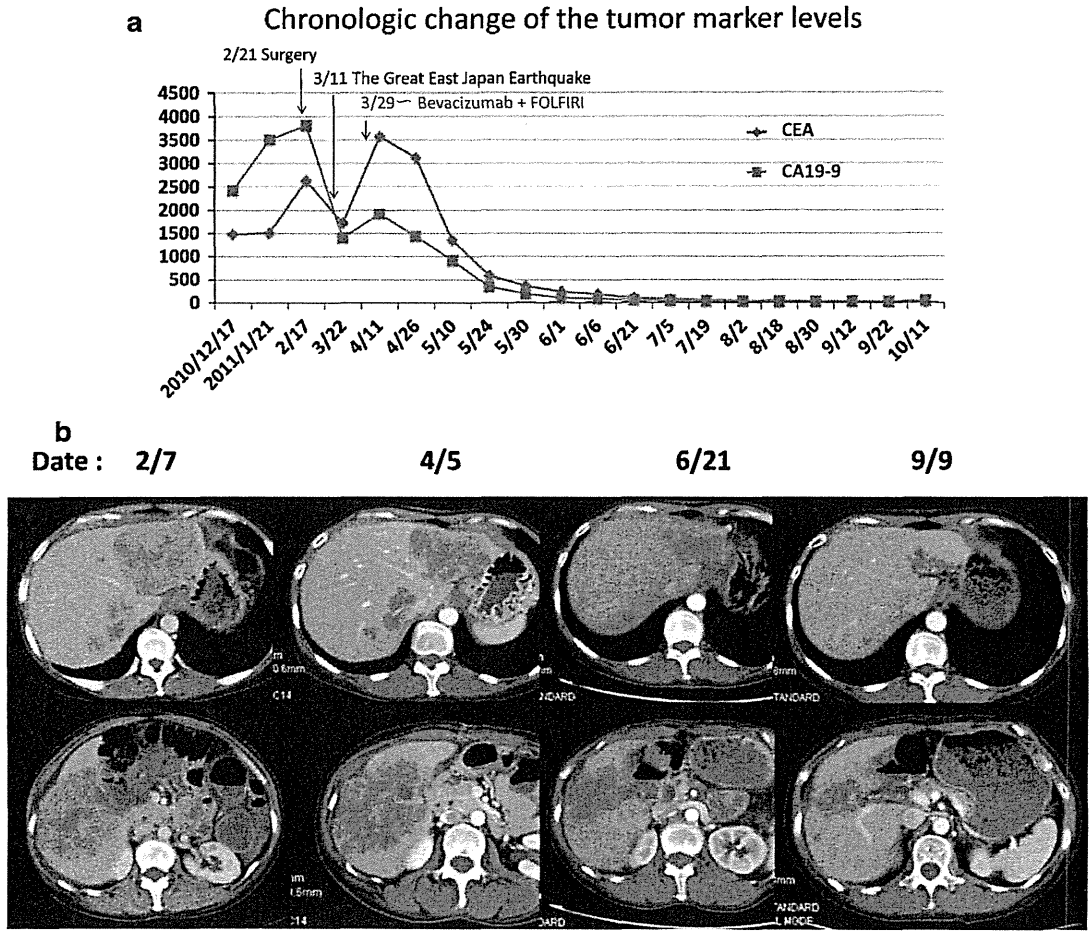

Fig. 8 Therapeutic effect of the systemic bevacizumab + FOLFIRI therapy. **a** Chronological change in the tumor marker levels. They decreased dramatically after administration of the systemic bevacizumab + FOLFIRI therapy. **b** Chronological change in liver metastases. They have decreased dramatically after administration of systemic bevacizumab + FOLFIRI therapy and have been in the PR state over a year

with DSBs repair, such as *MRE11* and *RAD50*. Taken together, MMR-deficient cells exhibit high sensitivity to CPT-11 [9–17]. Hence, CPT-11 treatment might be effective in patients with microsatellite instability-high (MSI-H) colorectal cancer.

Dr. M. Takahashi (medical oncologist): In fact, a previous randomized study for adjuvant chemotherapy against stage III MSI-H colon cancers demonstrated the significant advantage of the addition of CPT-11 [12]. However, another subsequent study did not reveal the benefit in a similar population [13]. Thus, the clinical benefit of CPT-11 compared with other agents in MSI-H colorectal cancer remains controversial. Because the mutations in *MRE11* or *RAD50* are detected in many but not all MSI-H tumors (70–85 %) [22], the MSI-H phenotype may not always correlate with the hypersensitivity of tumors to CPT-11. Another marker to indicate *MRE11* or *RAD50* deficiency (e.g., mutational analysis and/or IHC) may help to predict the efficacy of the CPT-11 treatment.

Dr. Ishioka: As both the former trials (CALGB89803 and PETACC-3) were adjuvant trials for stage II/III colorectal cancer [12, 13, 23], the clinical relevance of the outcome was slightly different in this stage IV case. While the evidence level of the advantage of CPT-11 for MSI-H tumor was not sufficient, selecting FOLFIRI among the standard therapies was reasonable according to the concept that the most promising therapy should be given priority. Moreover, when you use CPT-11, you must evaluate the patient for the *UGT1A1* gene polymorphism. Dr. Akiyama, please offer a general explanation.

Dr. Akiyama (medical oncologist): CPT-11 is inactivated by UGT1A1. If a specific gene polymorphism exists in *UGT1A1*, the glucuronidation level of SN-38, the active metabolite of CPT-11, would be attenuated, resulting in

drug accumulation and toxicity enhancement; this leads to diarrhea, neutropenia, etc. To be more precise, homozygosity for *UGT1A1*28* or *UGT1A1*6* and heterozygosity for both *UGT1A1*6* and *UGT1A1*28* are the polymorphisms mentioned in the package insert of the drug. However, optimal criteria for dosage adjustments have not been established. Moreover, there are some differences among ethnicities. In Asians, *UGT1A1*6* is more frequent than *UGT1A1*28*. Conversely, *UGT1A1*28* is much more common than *UGT1A1*6*, which is quite rare in Caucasians and African-Americans. Such discordance is derived from the different genetic background among the races [24–26].

Dr. Ishioka: With regard to dose, 150 mg/m^2 is defined as the maximal dose in Japan, although 180 mg/m^2 is the standard in Europe and the US. Accordingly, data from overseas cannot be used for direct comparisons. Many research groups, including ours, are working on this topic, and an appropriate criterion for the Japanese people needs to be established. Well, let us get back to this case. What about MSI in this case? Would you explain the reason, if you did not check?

Dr. Shiono: We obtained positive IHC results, and therefore, we did not perform an MSI examination. IHC is the best initial examination because it directs the candidate gene for subsequent mutation analysis in families with a high probability of having a mutation (the revised Bethesda guidelines or Amsterdam II criteria) [27]. Moreover, the latest analysis on the accuracy and cost-effectiveness of IHC and/or MSI examination to screen for Lynch syndrome [28] promotes the following strategies: "IHC and MSI performed simultaneously" and "IHC followed by MSI if IHCs were normal." The latter was slightly better in terms of cost. Therefore, IHC seems to be sufficient if it is performed first. According to this strategy, if IHC demonstrated the candidate mutated gene, you can skip MSI and proceed to direct sequencing. IHC has an advantage in terms of specifying the putative mutated MMR gene compared with MSI [29].

Dr. Ishioka: OK, so it is reasonable. However, how do you explain the discrepancy between the results of IHC and sequence analyses?

Dr. Shiono: As seen in Fig. 7b, nuclear staining of MSH6 alone was lost compared with that of the other three MMRs. Some possibilities were considered. For example, it is known that genomic rearrangements such as large deletions cannot be detected by conventional sequence analysis [30–32], actually in a significant proportion of Lynch syndrome families (5–20 %) [33–36]. Otherwise, it may be a type of epigenetic modification such as methylation [37]. However, further molecular analyses, e.g., the multiplex ligation-dependent probe amplification (MLPA) test, are needed for elucidation [31, 33, 38].

Dr. Ishioka: What about care for the families because this is a hereditary syndrome?

Dr. Shiono: Complying with the guidelines [39, 40], we performed a genetic counseling series for the patient, and her sister wished to accompany her. Her siblings shared the information, recognized the importance of medical follow-up, and have begun to undergo annual screening examinations, including colonoscopy. You can refer to the surveillance recommended by the international collaborative groups [27, 41]. However, a study indicated that the screening recommendations for *MSH6* mutation carriers may slightly differ from those for Lynch syndrome carriers as a whole, reflecting the characteristics of *MSH6*-mutated Lynch syndrome [42, 43]. The weaker phenotype, which is observed as a result of *MSH6* mutations, exhibits a later age of onset and lower penetrance compared with that observed as a result of *MLH1* or *MSH2* mutations [44, 45]. Many types of cancer should be considered in regard to an increased risk, e.g., cancer of the colorectum, endometrium, stomach, small intestine, hepatobiliary system, kidney, urinary bladder, brain, and ovary. The latest prospective study showed that pancreatic and breast cancers had an elevated risk [46].

Dr. Ishioka: Finally, what is the discriminative point in this case compared with other Lynch syndrome cases?

Dr. Shiono: In general, approximately 90 % of Lynch syndrome cases with mutations in any MMR genes are attributed to *MLH1* or *MSH2* mutations with distinct clinical features such as early onset (<50 years) and proximal colon predominance [47–52]. In contrast to these characteristics, it might have been difficult to diagnose Lynch syndrome in this case without a definitive family history. Moreover, with respect to comorbid cancer, while the frequency of endometrial cancer is as high as 60–70 %, the frequency of ovarian cancer is only 7–10 % [53]. Hence, clinical information on ovarian lesions might be relatively less likely to indicate Lynch syndrome. As mentioned above, although the incidence of Lynch syndrome attributed to *MSH6* mutation is as low as approximately 10 %, it is known to show "relatively late onset" and "low penetrance" propensity compared with *MLH1* or *MSH2* mutations [42, 44, 45]. Thus, judging by only clinical manifestation may lead to a diagnostic pitfall. To avoid misdiagnosis of Lynch syndrome, considering a family history is always critically important.

Dr. Shimodaira: Concerning the unique phenotype of MSH6-deficient Lynch syndrome, the mechanism can be understood when the molecular function of the four MMR proteins is considered. First, they function as heterodimers formed by MSH2 in association with MSH6 (MutSα) or MSH3 (MutSβ) and MLH1 interaction with PMS2 (MutLα), respectively. As seen in these complexes, the contribution of MSH6 is relatively small compared with that of major players such as MLH1 or MSH2, which

interact with many gene products. In fact, MSH6 functionally participates only in detection of single-base mismatch or small loop-out mutations, while MLH1 or MSH2 engages in widespread mismatches other than single-base abnormalities. Thus, the loss of MSH6 function involves only a partial deficiency of the MMR system, and subsequently it results in an attenuated clinical phenotype, which is approximated to conventional colorectal cancers with regard to late onset and low penetrance, compared with those caused by MLH1 or MSH2 deficiency [54, 55].

Dr. Shiono: In conclusion, this was a very intriguing discussion on diagnostics, treatment, pathology, and molecular biology. It is also dramatic that she was saved from the tsunami, which deprived her of her house on the seashore, by an occasional visit to our hospital during the Great East Japan Earthquake. She has also been spared from life-threatening disease progression by treatment based on the cooperation of many doctors. I appreciate all your kind collaborative work.

Dr. Ishioka: The first-line bevacizumab + FOLFIRI treatment exerted a pronounced effect on this metastatic case of *MSH6*-mutated Lynch syndrome. The population of Lynch syndrome cases with metastasis is too small to organize a large-scale randomized prospective trial in order to elucidate CPT-11 effectiveness. However, the prevalence of MSI among all colorectal cancers is approximately 15 % [56, 57]. Therefore, it might be possible to conduct a clinical trial by alternatively targeting similar types of cancers. Thus, further analysis is needed to elucidate the clinical benefit of the drug. Are there any questions? Then, this conference is adjourned. Thank you for your attendance.

What we learned from this case conference

1. You must always collect detailed information regarding family history in order not to overlook familial tumor syndromes.
2. You should know that weaker phenotypes, such as "late onset" and "low penetrance," compared to *MLH1*- or *MSH2*-mutated Lynch syndrome, can be observed because of *MSH6* deficiency.
3. A histopathological diagnosis must be obtained as soon as possible before deciding on an optimal regimen for patients with multiple primary cancers.
4. Although it is controversial at the clinical level and requires further study, a CPT-11-based regimen may have favorable effects on Lynch syndrome cases, depending on MMR deficiency.

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ORIGINAL ARTICLE

Clinical usefulness of *KRAS*, *BRAF*, and *PIK3CA* mutations as predictive markers of cetuximab efficacy in irinotecan- and oxaliplatin-refractory Japanese patients with metastatic colorectal cancer

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Abstract
Background Anti-epidermal growth factor receptor (EGFR) antibodies, cetuximab, and panitumumab are established as a new treatment option for metastatic colorectal cancer (mCRC). Among activating mutations downstream of EGFR, the *KRAS* mutation, which is present in 30–45 % of CRC patients, has shown to be a predictive biomarker of resistance to anti-EGFR antibody therapy based on Caucasian studies.
Methods Forty-three chemotherapy-refractory Japanese patients with mCRC were treated with cetuximab monotherapy or cetuximab plus irinotecan. *KRAS*, *BRAF*, and *PIK3CA* mutational status of tumors was assessed. The association between mutational status and treatment outcome was evaluated.
Results Of 43 tumors, *KRAS*, *BRAF*, and *PIK3CA* mutations were identified in 12 (27.9 %), 2 (4.7 %), and 2 (4.7 %) tumors, respectively. The wild-type *KRAS* subgroup showed better clinical outcomes than the mutant *KRAS* subgroup in terms of response rate (RR) (31.3 % vs. 0 %, $P = 0.034$) and progression-free survival (PFS) (5.1 vs. 3.0 months, $P = 0.017$). No responder to treatment was shown in 16 (37.2 %) patients with tumors harboring mutations in any one of the three genes (*KRAS*, *BRAF*, and *PIK3CA*). The wild-type subgroup without any mutations in *KRAS*, *BRAF*, and *PIK3CA* had a better RR (37.0 %) and PFS (6.4 months) than did the wild-type *KRAS* subgroup.
Conclusion Our data indicated that *KRAS* status is predictive of cetuximab response in the Japanese population. The additional analysis of *BRAF* and *PIK3CA* genes in wild-type *KRAS* patients could improve selection of patients who are most likely to benefit from anti-EGFR antibody therapy.

Keywords Cetuximab · Colorectal cancer · *KRAS* · *BRAF* · *PIK3CA*

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Introduction

Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, triggers a downstream signaling cascade through such as the RAS–RAF–MAPK and PI3K–AKT pathways, which are involved in cell proliferation, survival, and motility. Inhibition of EGFR activation has

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demonstrated significant promise as a molecular targeting therapy for various solid tumors. Two monoclonal antibodies (mAbs) targeting EGFR, cetuximab and panitumumab, have been approved for treatment of metastatic colorectal cancer (mCRC). The initial candidate biomarker for the anti-EGFR antibody response, EGFR expression analyzed by immunohistochemistry, was not a reliable predictive factor [1]. *KRAS*, downstream of EGFR, was shown to be a useful biomarker because somatic mutations that mainly occur in codons 12 and 13 result in constitutive activation of the RAS–MAP pathway regardless of EGFR inhibition [2–4]. A number of groups undertook retrospective *KRAS* testing of tumors from mCRC patients who were treated with cetuximab or panitumumab [5, 6]. Studies of patients receiving first and subsequent lines of treatment have found that those with mutated *KRAS* do not respond to, or experience any survival benefit from, treatment with anti-EGFR mAb [2–4, 6–10]. However, only a small proportion of patients achieved an objective response and benefit from cetuximab even among those with wild-type *KRAS* tumors. Thus, other downstream factors in EGFR signaling are now being explored, such as *BRAF* and *PIK3CA*, which are mutated in 5–10 % and 10–30 % of CRC, respectively.

Activating mutations in *BRAF*, which is mutually exclusive with *KRAS* mutations, may be responsible for the lack of efficacy of anti-EGFR mAbs in wild-type *KRAS* tumors [11, 12]. Retrospective analyses of anti-EGFR mAb-based treatment in various lines showed a correlation between the *BRAF* V600E and resistance to anti-EGFR mAb [11, 13]. *BRAF* mutation also has been shown to be both a prognostic factor and predictive of cetuximab response [13]. Therefore, interpretation of the clinical significance of *BRAF* mutations is complicated. The *PIK3CA* gene encodes the catalytic subunit p110α of PI3K. Tumor-derived mutant PI3K stimulates the AKT pathway and promotes cell growth in several cancers, including CRC. Tumors with *PIK3CA* mutations are associated with poor prognosis. Mutations in the *PIK3CA* gene have been shown to significantly impair response to treatment with anti-EGFR mAbs in mCRC patients. However, recent contradictory evidence indicates no strong rationale for using *PIK3CA* mutations as a single predictive marker for cetuximab response in chemotherapy-refractory mCRC [14]. A large-scale European study reported that the combination of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutation status improved prediction sensitivity for anti-EGFR mAb response [15].

The epidermal growth factor receptor is a critical predictive marker of gefitinib efficacy in non-small cell lung cancer (NSCLC). A clear ethnic difference in the frequency of EGFR mutations was found between Caucasians and Asians. The mutation frequency is higher in Asian NSCLC patients (about 30–60 %) than in Caucasian patients (approximately 10–20 %) [16–18]. However, the ethnic differences between Caucasians and Asians in mutation prevalence of *KRAS*, *BRAF*, and *PIK3CA* in mCRC have not been evaluated fully. Moreover, *KRAS* mutation status and that of other EGFR-downstream genes should be validated as predictive markers of anti-EGFR therapy in the Asian population.

We evaluated the relationship between *KRAS* mutation status and response to cetuximab-based treatment in Japanese patients with mCRC who have failed prior chemotherapy including irinotecan, oxaliplatin, and fluoropyrimidine. Furthermore, to optimize the selection of patients who are most likely to benefit from anti-EGFR mAbs, we investigated the association of minor *KRAS* mutations in codon 61, *BRAF* V600E mutation, and *PIK3CA* mutations in exons 9 and 20 with clinical outcomes.

Materials and methods

Patients and trial design

This study, aimed to examine the effect of cetuximab on RR and PFS among patients with mCRC in whom all prior chemotherapy had failed and for whom no other standard anticancer therapy was available, was approved by the Ethical Committee of Tohoku University School of Medicine. Eligible patients were enrolled between October 2008 and May 2010. Tumor specimens of all patients exhibited EGFR expression in >1 % of malignant cells, as determined by immunohistochemistry with the Dako EGFR PharmDx kit (DakoCytomation, Glostrup). None of the patients had received previous treatment with anti-EGFR mAb. After enrollment, patients received cetuximab-based treatment. Cetuximab was administered intravenously at a standard dosage of 400 mg/m^2 over 2 h on day 1 of treatment, followed by 250 mg/m^2 intravenously over 1 h, once a week. Irinotecan was administered intravenously at a standard dosage of 150 mg/m^2 every 2 weeks or 100 mg/m^2 weekly for 3 consecutive weeks, following by a 1-week rest. Patients were evaluated for tumor response or progression every 8 weeks by radiologic imaging. Cetuximab-based treatment was continued until disease progression or unacceptable toxicity occurred.

Tumor collection and processing

Formalin-fixed, paraffin-embedded (FFPE) samples of tumor tissue from archival specimens collected at the time of diagnosis were stored at Tohoku University Hospital. Assays of tissue samples for *KRAS*, *BRAF*, and *PIK3CA*

mutations were performed at the Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University. All patients' samples were screened for *KRAS* mutation in codons 12, 13, and 61, and for *BRAF* V600E and *PIK3CA* mutations in exons 9 and 20. All available tissue samples were classified as mutant or wild type.

Nucleotide sequence analysis

Mutation analyses of *KRAS*, *BRAF*, and *PIK3CA* were performed by extraction of genomic DNA from FFPE tissue slides or sections. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's protocol. Analyses of the DNA sequences were performed with the use of the automated CEQ2000XL DNA analysis system (Beckman Coulter) under specific cycle and temperature conditions. The PCR products were analyzed by 1.0 % agarose gel electrophoresis. Appropriate positive and negative controls were included for *KRAS*, *BRAF*, and *PIK3CA*. To minimize bias, the persons who performed the mutation analyses were blinded to clinical outcomes.

Statistical analysis

All patients for whom data on *KRAS*, *BRAF*, and *PIK3CA* mutation status were available were included in the analysis. The statistical analyses of categorical variables were performed using the χ^2 test. RR was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST) ver. 1.0. According to RECIST criteria, patients were categorized as responders if they achieved complete response (CR) or partial response (PR), or nonresponders if they showed stable disease (SD) or progressive disease (PD). PFS was defined as the time from the beginning of chemotherapy until the first objective evidence of disease progression or death from any cause. The PFS analyses were determined according to the Kaplan–Meier method, and survival curves were compared using the log-rank test. Statistical significance was set at $P < 0.05$ for a bilateral test.

Table 1 Patient characteristics

	All	*KRAS* mutant	*KRAS* wild type
Total number of patients	43	12	31
Median age, years (range)	57 (31–80)	56 (41–80)	63 (31–79)
Gender			
Male	25	6	19
Female	18	6	12
ECOG performance status			
0	29	10	19
1	13	2	11
2	1	0	1
Number of previous chemotherapy lines			
1	0	0	0
2	25	8	17
≥3	18	4	14
Prior chemotherapy for advanced disease			
FOLFOX	43	12	31
FOLFIRI/IRIS/Irinotecan/IFL	33/5/3/2	10/1/0/1	23/4/3/1
Bevacizumab	17	4	13
Chemotherapy regimen			
Cetuximab + irinotecan	31	12	19
Cetuximab alone	12	0	12
Primary tumor			
Cecum	2	1	1
Ascending colon	8	3	5
Transverse colon	3	1	2
Descending colon	0	0	0
Sigmoid colon	12	2	10
Rectum	18	5	13
Metastatic sites			
Liver	32	9	23
Lung	27	8	19
Intraabdominal lymph nodes	15	2	13
Peritoneum	7	2	5
Bone	3	0	3
Others	6	2	4

FOLFOX 5-fluorouracil, leucovorin, oxaliplatin, *FOLFIRI* 5-fluorouracil, leucovorin, irinotecan, *IRIS* irinotecan, S-1, *IFL* irinotecan, 5-fluorouracil, leucovorin

Results

Patient characteristics

Patient clinical characteristics are listed in Table 1: 43 patients received cetuximab-based treatment. Of these, 42 patients were ECOG performance status 0 or 1, and only 1 patient was ECOG performance status 2.

All patients had failed prior chemotherapy including irinotecan, oxaliplatin, and fluoropyrimidine. None of the patients had been treated with anti-EGFR mAbs. Prior oxaliplatin-containing regimen included only the FOLFOX regimen [infusion and bolus 5-fluorouracil (5-FU) plus oxaliplatin]. Prior irinotecan-containing therapies included the FOLFIRI regimen (infusion and bolus 5-FU with irinotecan) in 33 patients, irinotecan monotherapy in 3

patients, S-1 plus irinotecan in 5 patients, and the IFL regimen (bolus 5-FU plus irinotecan) in 2 patients. Seventeen patients received bevacizumab in their treatment regimen.

The sites of metastases were liver (32; 74.4 %), followed by lung (27; 62.8 %), intraabdominal lymph nodes (15; 34.9 %), and peritoneum (7; 16.3 %). Among 43 patients with mCRC, 31 (72.1 %) received cetuximab plus irinotecan and 12 (27.9 %) received cetuximab monotherapy.

Toxicity

Toxicity data are summarized in Table 2. Grade 3–4 neutropenia was observed in 12 patients (27.9 %), and grade 3–4 anemia was observed in 4 (9.3 %). Skin toxicity, including acne, rash, dry skin, pruritus, acneiform dermatitis, and papular rash, was observed in 42 (97.7 %) patients. Grade 3–4 skin toxicity was observed in 4 patients (9.3 %). Other grade 3–4 toxicities included diarrhea (2.3 %), stomatitis (2.3 %) and hypomagnesia (2.3 %). The toxicity profiles did not differ between patients with wild-type *KRAS* tumors and those with mutated *KRAS* tumors.

Mutation analyses of *KRAS*, *BRAF*, and *PIK3CA*

Table 3 provides a list of mutations detected by direct sequencing. We analyzed a relatively rare mutation in codon 61 in addition to the common mutations in codons 12 and 13 to increase the sensitivity of mutation detection. *KRAS* mutations at codons 12, 13, and 61 were observed in 12 (27.9 %) of the tumors. Of the 11 detected mutations in codons 12 and 13, the most frequent mutation was G12D (14.0 %), followed by G13D (7.0 %), G12V (2.3 %), and G12A (2.3 %). Q61H was found in 1 tumor (2.3 %). Two of the three common *KRAS* mutations, G12D, G13D, and G12V, were also detected frequently in this study. *BRAF* mutation at codon 600 (V600E) was observed in 2 tumors (4.7 %), both of which were *KRAS* wild type. *PIK3CA* mutations in exon 9 (E542K and E545G) were observed in 2 patients (4.7 %), but no tumor mutations were found in exon 20.

Table 2 Toxicity profile in 43 mCRC patients

Event	All ($n = 43$)		*KRAS* mutant ($n = 12$)		*KRAS* wild type ($n = 31$)	
	G1–4 (%)	G3–4 (%)	G1–4 (%)	G3–4 (%)	G1–4 (%)	G3–4 (%)
Leukopenia	16 (37.2)	5 (11.6)	4 (33.3)	2 (16.7)	12 (38.7)	3 (9.7)
Neutropenia	18 (41.9)	12 (27.9)	4 (33.3)	4 (33.3)	14 (45.2)	8 (25.8)
Anemia	11 (25.6)	4 (9.3)	1 (8.3)	0 (0)	10 (32.3)	4 (12.9)
Thrombocytopenia	2 (4.7)	0 (0)	1 (8.3)	0 (0)	1 (3.2)	0 (0)
Diarrhea	11 (25.6)	1 (2.3)	1 (8.3)	0 (0)	10 (32.3)	1 (3.2)
Skin toxicity	42 (97.7)	4 (9.3)	12 (100)	1 (8.3)	30 (96.8)	3 (9.7)
HFS	8 (18.6)	0 (0)	1 (8.3)	0 (0)	7 (22.6)	0 (0)
Stomatitis	15 (34.9)	1 (2.3)	4 (33.3)	0 (0)	11 (35.5)	1 (3.2)
Nausea	12 (27.9)	0 (0)	2 (16.7)	0 (0)	10 (32.3)	0 (0)
Vomiting	5 (11.6)	0 (0)	0 (0)	0 (0)	5 (16.1)	0 (0)
Fatigue	16 (37.2)	0 (0)	3 (25.0)	0 (0)	13 (41.9)	0 (0)
Anorexia	10 (23.3)	0 (0)	2 (16.7)	0 (0)	8 (25.8)	0 (0)
Hypomagnesia	11 (25.6)	1 (2.3)	1 (8.3)	0 (0)	10 (32.3)	1 (3.2)

HFS hand–foot syndrome

Table 3 *KRAS*, *BRAF*, and *PIK3CA* mutation frequencies ($n = 43$)

Gene	Codon	Nucleotide substitution	Amino acid substitution	Number (%)	
KRAS	12	GGT → GAT	G12D	6 (14.0)	12 (27.9)
		GGT → GCT	G12A	1 (2.3)	
		GGT → GTT	G12V	1 (2.3)	
	13	GGC → GAC	G13D	3 (7.0)	
	61	CAA → CAC	Q61H	1 (2.3)	
BRAF	600	GTG → GAG	V600E	2 (4.7)	2 (4.7)
PIK3CA	542	GAA → AAA	E542K	1 (2.3)	2 (4.7)
	545	GAG → GGG	E545G	1 (2.3)	

Table 4 Response to cetuximab according to the presence or absence of gene mutations in the 43 patients

Tumor response	*KRAS* status in codons 12, 13		Genetic status of *KRAS* (codons 12, 13, 61), *BRAF*, and *PIK3CA*		All patients
	Mutant (%)	Wild type (%)	Mutant of any genes (%)	Wild type of all genes (%)	
Total	11 (100)	32 (100)	16 (100)	27 (100)	43 (100)
CR	0 (0)	1 (3.1)	0 (0)	1 (3.7)	1 (2.3)
PR	0 (0)	9 (28.1)	0 (0)	9 (33.3)	9 (20.9)
SD	7 (63.6)	11(34.4)	8 (50.0)	10 (37.0)	18 (41.9)
PD	4 (36.4)	11 (34.4)	8 (50.0)	7 (25.9)	15 (34.9)
RR (%)	0	31.3	0	37.0	23.3
DCR	63.6	65.6	50.0	74.1	65.1
PFS (median)	3.0 M	5.7 M	2.8 M	6.4 M	4.7 M

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *M* months

Cetuximab efficacy

The RR and median PFS (mPFS) according to the presence or absence of gene mutations are shown in Table 4. In the 43 assessable patients, the RR and mPFS correlated with *KRAS*, *BRAF*, and *PIK3CA* mutation status. No responder was observed among the 16 patients with mutations in any one of the three genes, although there were 11 responders among the 27 patients with no gene mutation. In the 27 patients with no detected mutations, objective RR was 40.7 %; in 16 patients with mutated tumors, objective RR was 0 %. In patients with wild-type *KRAS* in codons 12 and 13, *KRAS* in codon 61, *BRAF*, and *PIK3CA* mutations were associated with lack of response.

The mPFS of the wild-type *KRAS* (codon 12 and 13) subgroup was significantly longer than that of mutant *KRAS* (codon 12 and 13) subgroup (5.7 vs. 3.0 months; $P = 0.017$) (Fig. 1a). However, the difference of mPFS between wild-type *KRAS* (codon 12, 13, and 61), *BRAF* and *PIK3CA* subgroup, and mutant subgroup in any of the three genes was considerably more (6.4 vs. 2.8 months; $P = 0.0069$) (Fig. 1b). Consistent results with RR and mPFS were observed in the plot of best response of target lesions and mutation status. Almost all patients with any mutation in *KRAS*, *BRAF*, and *PIK3CA* failed to respond to cetuximab-based treatment (Fig. 2a). No patient in the mutant *KRAS* group had a tumor reduction (Fig. 2b). In contrast, 50 % of the wild-type *KRAS* group had a tumor reduction, including patients with PR and SD (Fig. 2c); 0.06 % of the group with any mutant *KRAS*, *BRAF*, and *PIK3CA* and 56 % of the all wild-type group had a tumor reduction, respectively (Fig. 2d, e). All the four patients with severe progressive disease (more than 40 % tumor increase from baseline) were included in the group with any mutant *KRAS*, *BRAF*, and *PIK3CA* genes. These results indicate the clinical relevance of mutations in these genes in predicting the efficacy of cetuximab-based treatment in patients with mCRC.

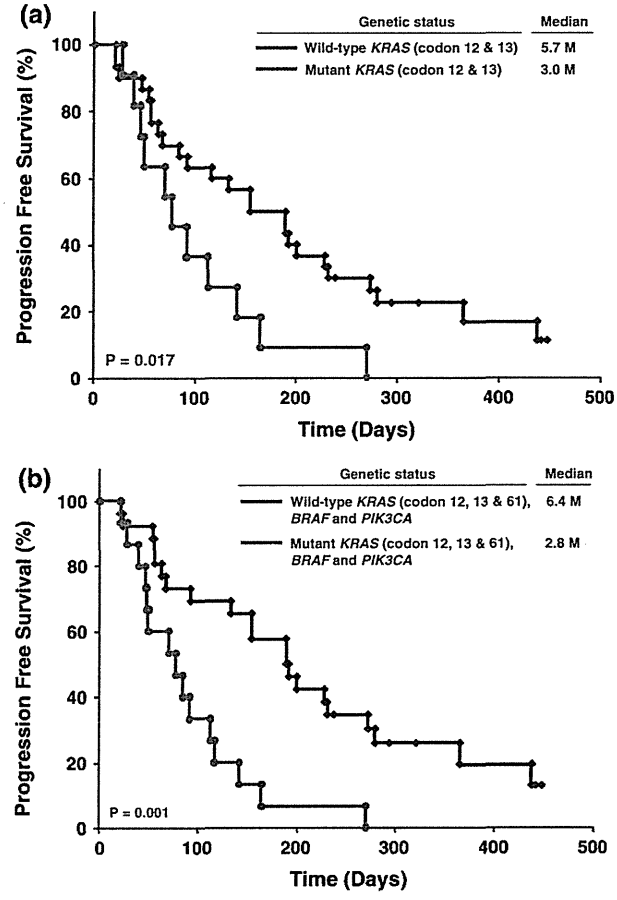

Fig. 1 Kaplan–Meier cumulative progression-free survival (PFS) based on *KRAS*, *BRAF*, and *PIK3CA* mutational status in metastatic colorectal cancer (mCRC) patients treated with cetuximab. **a** Patients with wild-type *KRAS* (codons 12, 13) versus mutant *KRAS*. **b** Patients with all wild-type *KRAS* (codons 12, 13, 61), *BRAF*, and *PIK3CA* versus any mutant *KRAS*, *BRAF*, and *PIK3CA*

Discussion

Our data confirmed that *KRAS* status is a significant predictive marker of cetuximab response in Japanese patients

with mCRC as it is in Caucasians, and the combination of *KRAS*, *BRAF*, and *PIK3CA* analyses improved predictive sensitivity. The wild-type *KRAS* (codons 12 and 13) subgroup showed better clinical outcomes than did the mutant *KRAS* subgroup in terms of RR and mPFS (Fig. 1a). Moreover, the difference of clinical outcome was wider by comparing between the wild-type subgroup in all *KRAS* (codons 12, 13, and 61), *BRAF*, and *PIK3CA* genes and the mutant subgroup in any of the three genes than comparing between the wild-type *KRAS* (codons 12 and 13) and the mutant subgroup (Fig. 1b). Then, combined analysis of the three genes and addition of *KRAS* codon 61 mutation analysis contributed to a better selection of the patients likely to benefit from cetuximab treatment. In contrast, no responders were found among the five patients with tumors harboring either *KRAS* codon 61, *BRAF*, or *PIK3CA* mutations. It is a noteworthy tendency that combination of mutations of the three genes contributes to selecting severely progressive patients who benefit least from anti-EGFR therapy (Fig. 2a). The RR of the wild-type *KRAS* and the RR of the wild-type *KRAS*, *BRAF*, and *PIK3CA* in this study were almost comparable with those of the large-scale analysis in Europeans [15], suggesting that the significance of *KRAS*, *BRAF*, and *PIK3CA* mutations in prediction of cetuximab efficacy is almost identical between Asians and Caucasians. Nevertheless, almost 60 % of patients without any mutations in *KRAS*, *BRAF*, and *PIK3CA* genes still did not respond to cetuximab and suffered tumor progression. These results also suggest that there are other, unidentified molecular response determinants. We analyzed other downstream factors in the EGFR signaling pathway including *NRAS*, *AKT1*, and *PIK3R1*. Although previous reports have shown mutations in *NRAS*, *AKT1*, and *PIK3R1* genes in 2.64 % [15], 6 % [19], and 8.3 % [20] of patients with mCRC, respectively, we did not identify any mutations in these genes. Thus, we could not evaluate the significance of these gene mutations as a biomarker of anti-EGFR therapy because of low prevalence. However, we excluded the possibility that these genes were responsible for the treatment resistance we observed in patients with *KRAS*, *BRAF*, and *PIK3CA* wild-type mCRC. Additional biomarkers are needed to improve the identification of patients who will benefit from cetuximab treatment. One of the candidate biomarkers is the tumor suppressor PTEN protein, which is a negative regulator of PI3-kinase-initiated signaling. The loss of PTEN expression determined by immunohistochemistry has been associated with a lack of response to cetuximab [21, 22].

The *KRAS* mutation frequency in this study was low (27.9 %) in comparison to previous reports (40–50 %). The reason for this lower prevalence is likely the result of clinical bias as a consequence of the retrospective study design. We enrolled patients who received cetuximab as third-line therapy or later just after approval of cetuximab for use in Japan. Initially, the patients were treated with cetuximab without *KRAS* analysis in advance, causing no bias in the population of the *KRAS* mutants. However, after the *KRAS* analysis became available, the patients were treated only if the tumors harbored wild-type *KRAS*. This situation made the mutation frequency of *KRAS* lower than other studies, but also made our data valuable because no further clinical data regarding cetuximab treatment in Japanese patients with *KRAS*-mutant tumors will be available. The *KRAS* mutation frequency in 186 patients with mCRC was also analyzed during this study, including patients who did not receive cetuximab treatment for various reasons. The *KRAS* mutation was found in this population in similar frequency to that described in the previous studies (75/186 = 40.3 %). Moreover, the pattern of *KRAS* mutations was very similar to the previous Caucasian studies [23, 24]. Thus, we concluded that *KRAS* mutation in terms of both frequency and the mutation spectrum does not differ between Japanese and Caucasians. Recently, the *KRAS* G13D mutation has been shown to be associated with better outcome after treatment cetuximab than was observed with other mutations [25]. In this study, three patients with *KRAS* G13D-mutated tumor had no tendency to show better response to cetuximab-based therapy than those with other mutations (Fig. 2c), even though the sample size was low. The prevalence of *BRAF* mutation (4.6 %) was also lower than the reports in Caucasian studies [26], which could be the result of ethnic difference. However, *BRAF* mutations have shown to be a prognostic marker and a predictive marker of anti-EGFR antibody therapy [13]. Then, one of the possible explanations of this lower prevalence is that patients with the *BRAF* mutation become intolerant of additional therapy through multiple lines of chemotherapy, as similarly reported in several studies [15]. The prevalence of *PIK3CA* mutation (4.7 %) was quite lower than that observed in the previous studies (10–20 %). Of the two detected mutations, E542K is one of the three hot-spot mutations (E542K, E545K, and H1047R), whereas E545G is a rare mutation [15, 27]. Large-scale analysis will clarify whether this discrepancy in mutation frequency and spectrum is caused by ethnic differences. The clinical relevance of *PIK3CA* mutations in prediction of the response to anti-EGFR therapy is still controversial. Although most studies do not evaluate the mutation in exons 9 and 20 separately, a recent large European study has shown that only *PIK3CA* mutations in exon 20 but not those in exon 9 are associated with resistance to anti-EGFR antibody. We detected the *PIK3CA* mutation only in exon 9, and the mutated tumor showed no response to cetuximab. Our data indicated the mutations in exon 9 possibly abrogated the effect of cetuximab.