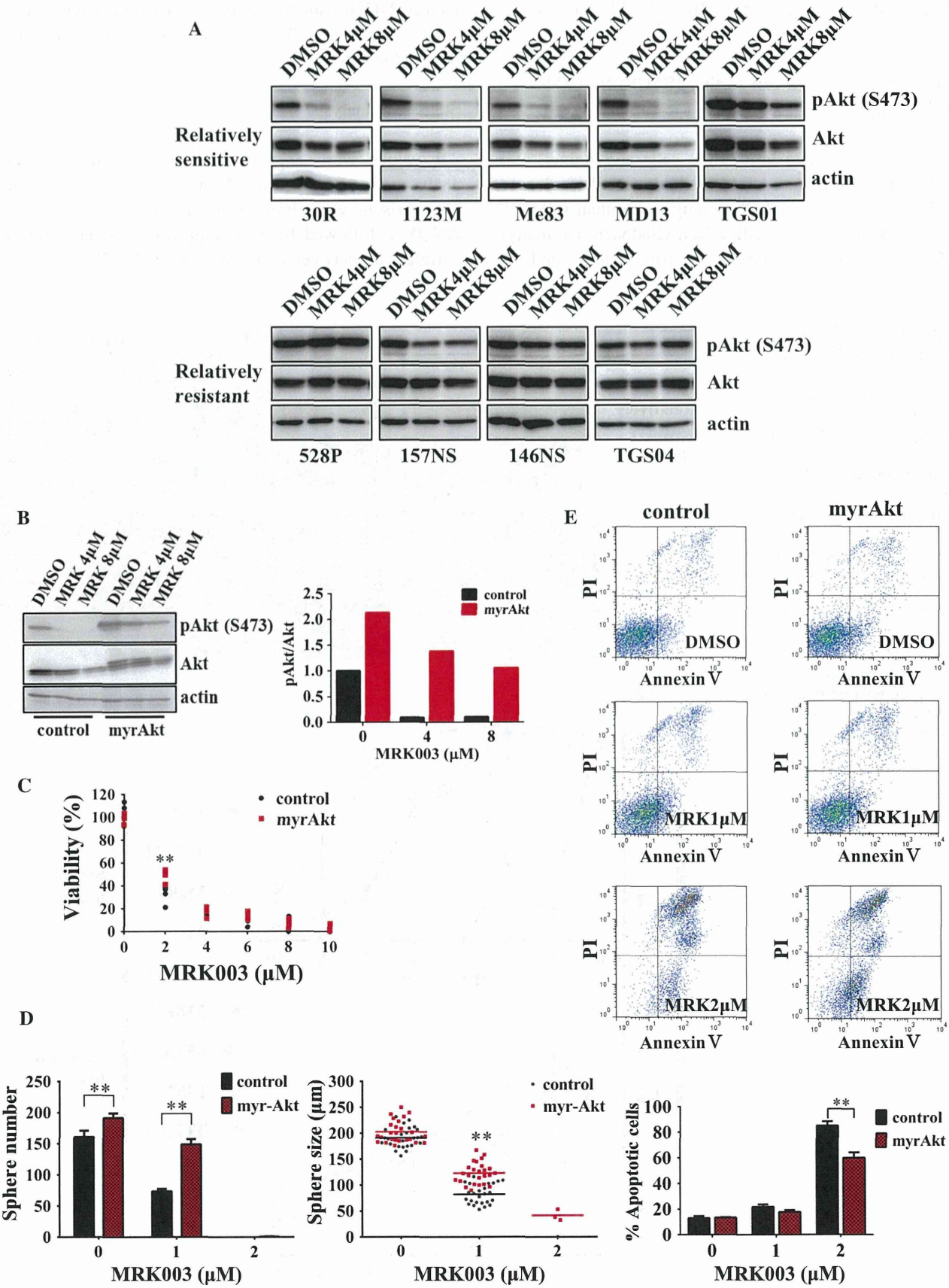
A
DMSO
MRK4μM
MRK8μM
Relatively sensitive
pAkt (S473)
Akt
actin
30R
1123M
Me83
MD13
TGS01
Relatively resistant
528P
157NS
146NS
TGS04
B
MRK 4μM
MRK 8μM
control
myrAkt
pAkt/Akt
MRK003 (μM)
C
Viability (%)
MRK003 (μM)
D
Sphere number
myr-Akt
Sphere size (μm)
% Apoptotic cells
E
PI
Annexin V
DMSO
MRK1μM
MRK2μM

◀**Fig. 4** Relatively sensitive GICs exhibited strong inhibition of the Akt pathway by MRK003. Response of MRK003 was not completely dependent on Akt. **a** GICs treated with MRK003 for 48 h were immunoblotted with phospho-Akt (ser473) and total-Akt. Actin was used as a protein loading control. **b** Relatively sensitive GIC, 30R was transfected with empty-vector (control) or myristoylated-Akt (myrAkt) vector. Western blot analysis of phospho-Akt and total-Akt was shown. The phosphorylation levels of Akt in control-30R and myrAkt-30R were quantified using ImageJ (1.45 s, National Institutes of Health, Bethesda, MD, USA) and shown as a diagram. Actin was used as loading control. The quantified level of Akt phosphorylation in control-30R with DMSO was considered as to be 1.0. **c** Viability of myrAkt-30R was only significantly increased than control-30R in 2 μM MRK003. **d** In tumorsphere-forming assay, myrAkt-30R sphere number and sphere-size were significantly increased compared to control-30R with 1 μM MRK003. Both myrAkt-30R and control-30R exhibited negligible sphere formation in 2 μM MRK003. **e** Apoptosis assay after MRK003 treatment for 72 h was analyzed by flow cytometry of AnnexinV-FITC. In 2 μM MRK003, the AnnexinV-FITC positive percentage of myrAkt-30R was about 60 %, was significantly decreased in comparison to control-30R. All data were representative. Experiments were conducted in at least triplicate. $^{**}P < 0.01$ using two-way ANOVA

PE and IC_{50} of MRK003 was analyzed using Spearman correlation test.

Results

MRK003 decreased cell viability of patient-derived GICs

We initially investigated the chemosensitivity of the nine GICs to MRK003 by using the viability assay (Fig. 1). MRK003 decreased viability in all species of GICs in a dose dependent fashion compared with DMSO control. Based on the above results we categorized these cells into two types: the former as relatively sensitive GICs and the latter as relatively resistant GICs. Viability of five relatively sensitive GICs (30R, 1123M, MD13, Me83 and TGS01) decreased dramatically with the use of less than 2 μM MRK003 compared to DMSO control. In contrast, viability of four relatively resistant GICs (528P, 157NS, 146NS, and TGS04) was reduced to half by the addition of more than 3 μM MRK003 compared to DMSO control.

MRK003 induced apoptosis in GICs

Subsequently, we assessed whether the reduction of viability by MRK003 was associated with the induction of apoptosis since the Notch signal was related to apoptosis regulation [10, 17]. As expected, MRK003 induced apoptosis in all species of GICs. In case of the sensitive GIC lines, even less than 4 μM MRK003 induced a significant increase in the apoptosis percentage as compared to the DMSO control (Fig. 2a, Supplementary Fig. 1a). However, in case of the resistant GIC lines, more than 8 μM MRK003 was required to induce an enhanced rate of apoptosis in comparison to the control (Fig. 2b, supplementary Fig. 1b). This assay demonstrated that even low concentrations of MRK003 were capable of inducing accelerated apoptosis in GICs and reducing their viability. These findings are parallel with the results of cell viability assay, suggesting that suppression of cell viability by MRK003 might be attributable to apoptosis.

MRK003 inhibited sphere formation of GICs

We employed the tumorsphere forming assay to analyze the role of MRK003 in GICs. The basal sphere forming abilities in the 9 GICs were rich in variety. Our results demonstrated that MRK003 significantly reduced both, sphere-number and -diameter in all species of GICs in a dose dependent manner (Fig. 3a, b). In the sensitive lines, both sphere-number and -diameter were significantly decreased with the use of up to 2 μM MRK003 ($P < 0.01$), and further, sphere formation was completely blocked by 3 μM MRK003. In the resistant lines, although sphere numbers and diameters were suppressed by 2, 4 or 6 μM MRK003, dramatic suppression of sphere formation was achieved with the use of more than 8 μM MRK003, compared with DMSO control ($P < 0.01$) (Fig. 3b). These data supported the observations that MRK003 impaired self-renewal in GICs. MRK003 sensitivity of GICs in the viability and apoptosis assays corresponded well with the sensitivity of the sphere forming assay.

Akt activation modestly improved the effect of MRK003

Notch signal is known to be associated with the subsequent activation of the PI3K/Akt pathway which is related to cell proliferation and the most major pathway in GBM [11, 15, 18, 19]. Therefore, phosphorylation level of Akt was assessed in all GICs after treatment with MRK003 for 48 h. Basal level of phospho-Akt in both the sensitive and the resistant lines were approximately same. It was observed that though MRK003 effectively decreased phospho-Akt level in a dose-dependent manner in the sensitive lines, it poorly affect the phosphorylation level of Akt in the resistant lines (Fig. 4a). The phospho-Akt level in sensitive lines might be caused that MRK003 could be due to the inhibition of total Akt production. Therefore, this data led us to hypothesize that dephosphorylation of Akt by MRK003 can explain the efficacy for the sensitive lines.

We further evaluated whether Akt was responsible for the effectiveness by MRK003 in the sensitive lines. To demonstrate that Akt activation rescues cells from the

effect of MRK003, myristoylated Akt vector was constitutively anchored at the membrane for maintaining it in an activated form, and was used for the transfection of the representative relatively sensitive GIC, 30R (myrAkt-30R). MyrAkt-30R exhibited higher levels of Akt activation than 30R transfected with empty vector (control-30R) (Fig. 4b). Expression of phospho-Akt was completely suppressed in control-30R by MRK003 treatment. In contrast, phospho-Akt levels in myrAkt-30R were maintained by MRK003 treatment, although a dose dependent decrease was observed (Fig. 4b).

In cell viability assay, the decrease of viability in control-30R by treatment with 2 μM MRK003 was reduced in myrAkt-30R ($P < 0.01$). However, rescue of viability was not significant in myrAkt-30R when treated with more than 4 μM MRK003 (Fig. 4c). It suggested that transfection with myr-Akt only partially abrogated the effect of MRK003. In the tumorsphere-forming assay performed with 1 μM MRK003, number and size were significantly increased in cells transfected with myrAkt-30R as compared to cells transfected with the control-30R ($P < 0.01$). However, when the concentration of MRK003 was increased to 2 μM, Akt induction hardly rescued tumorsphere formation (Fig. 4d). In consistence with the above results, Akt induction only partially suppressed apoptosis from 85.0 ± 3.4 % (mean ± SD) to 60.0 ± 4.2 % upon treatment with 2 μM MRK003 ($P < 0.01$) (Fig. 4e). Altogether, the effect of MRK003 in 30R could not be completely recovered with transfection of the cells with myrAkt. We observed similar results in 1123 M, another population of relatively sensitive GIC used in our study (supplementary Fig. 2a–d). Accordingly, the effect of MRK003 might be only partially dependent upon the inhibition of the Akt pathway.

CD44-high and CD133-low GICs were sensitive to MRK003 treatment

CD44 and CD133 were known as cancer stem cell surface markers [5, 38–40]. We analyzed the expression of CD44 and CD133 in the nine GICs. Our data indicated that sensitive lines presented high CD44 and low CD133 expression level, and that resistant lines exhibited high level expression of CD133 (Fig. 5a, b, supplementary Fig. 3a, b). We further investigated whether the expressions of CD44 and CD133 could serve as indicators of MRK003 sensitivity and analyzed the correlation between CD44 and CD133 expression levels and IC_{50} of MRK003. IC_{50} of GICs for MRK003 correlated negatively with CD44 expression ($P = 0.005$, $r = -0.865$), and positively with CD133 expression ($P = 0.037$, $r = 0.712$) (Fig. 5c). These findings indicated that the expression level of CD44 and CD133 may be useful as biomarkers for MRK003 efficacy.

Discussion

The data in the present study have demonstrated that MRK003 suppressed cell viability and sphere formation ability, and induced apoptosis in GICs derived from multiple patients. These results support effectiveness of MRK003 for GICs properties. Interestingly, GICs were divided into relatively sensitive GICs and relatively resistant GICs against MRK003. MRK003 sensitive and resistant GICs were demonstrated to be different by CD44 and CD133 expressed patterns. This study is the first to demonstrate that MRK003 might have strong therapeutic potential for CD44-high and CD133-low expressed GICs.

Although the downstream details of Notch signaling are yet to be deciphered, the Akt pathway which was crucial pathway in GBM had been known to be associated with Notch downstream signal [10, 13, 17, 20]. Our data demonstrated that phosphorylation of Akt in MRK003 relatively sensitive GICs was remarkably decreased by MRK003 treatment. However, transfection with constitutively active Akt vector could not negate the effect of MRK003 completely, thereby suggesting that the effect of MRK003 was only partially dependent on the inhibition of the Akt pathway. Notch signal is a major regulator of survival pathway to play a prominent role in bridging various pathways. Alternate cross-talk pathways for Notch signaling besides PI3K/Akt are TGF-β, MEK/ERK and JAK/STAT3 pathways [41, 42], which are also associated with cell proliferation in GBM [17, 20, 43]. The nature of Notch cross-talk system was flexible and was depended on each cell background [41]. Therefore, it is possible that MRK003 inhibits not only Notch signaling but also other pathways. Further investigations are warranted in this regard.

CD44 is reported to be related to tumor initiations and a novel therapeutic target [40, 43]. CD133 is a cell surface marker of cancer initiating cells in several cancer types although its validity remains controversial [5, 44]. In our findings, CD44-high and CD133-low expressed GICs are relatively sensitive to MRK003, whereas CD133-high expressed GICs are relatively resistant to MRK003. One explanation for chemoresistance to MRK003 is that CD133-high GICs may have potential drug resistance gene against chemotherapy. For example, CD133-high GICs possess high level of drug resistance gene, BCRP1 [45]. CD44-enriched GICs was regulated by TGF-β pathway which is a key regulator of malignant phenotype [35, 43, 46]. It may be hypothesized that the effect of Notch inhibition is exhibited partially via the TGF-β inhibition [43, 47]. GBM was segregated into four subtypes based on gene expression signature. CD44 was enriched in the mesenchymal subtype of GBM [48]. In our findings, GICs with high CD44 expression demonstrated to be very sensitive to

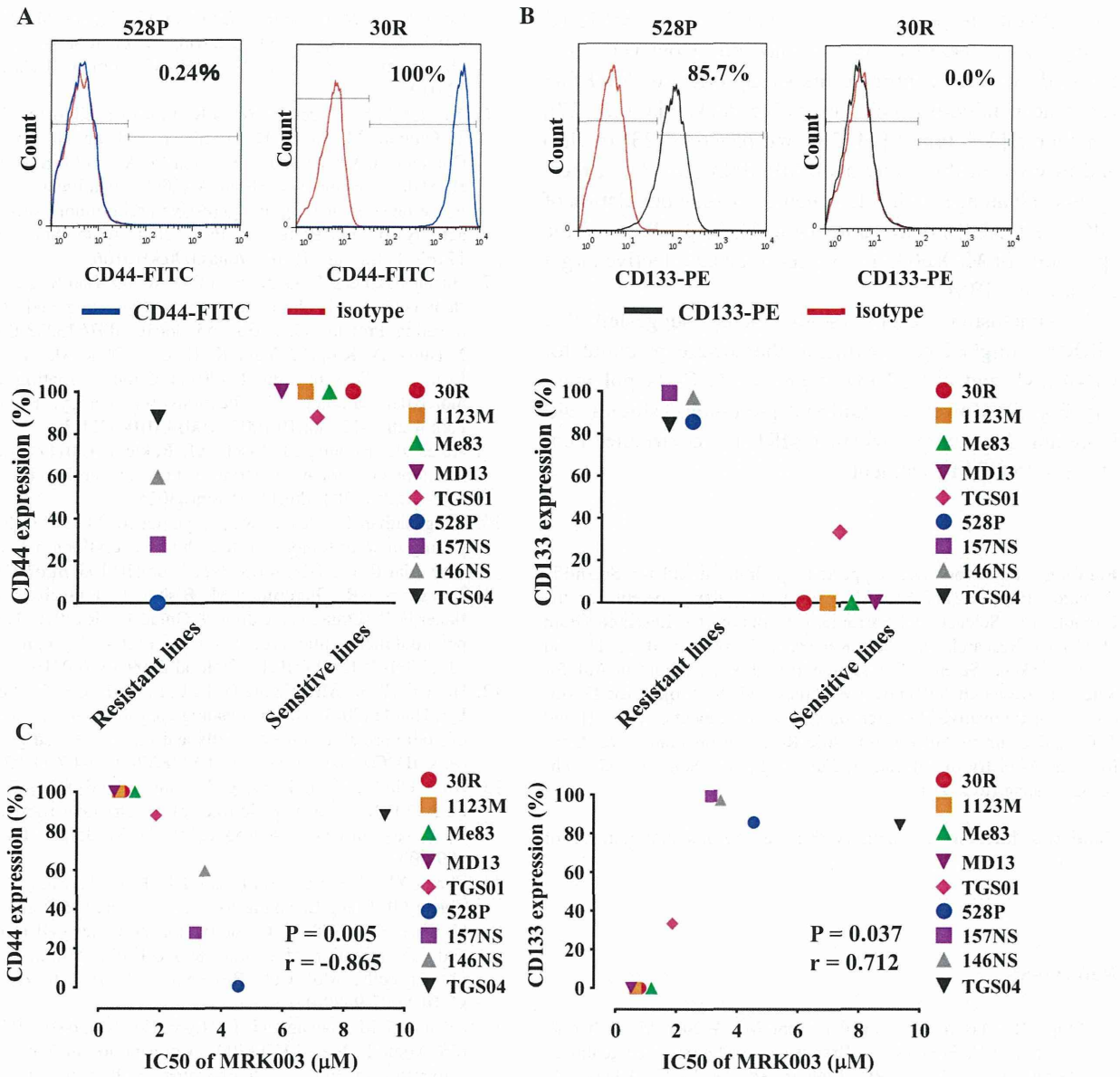

Fig. 5 MRK003 might be effective for CD44-high and CD133-low GICs. **a** *Sensitive lines* were high CD44-FITC positive percentage (range 87.9–100 %). On the other hand, *resistant lines* were broad CD44-FITC positive percentage (range 0.24–87.7 %). **b** *Sensitive lines* exhibited a low CD133-PE positive percentage (range 0–33.4 %). *Resistant lines* exhibited high CD133-PE positive percentage (range 84.2–99.4 %). **c** IC_{50} of MRK003 for GICs correlated to both CD44 and CD133 expression. Data shown was *scatter diagram* of nine GICs. X-axis was IC_{50} (μM) of MRK003. Y-axis was expression (%) of CD44 or CD133

MRK003. Therefore, MRK003 might be effective to mesenchymal subtype of GBM.

We performed sphere forming assay with other γ-secretase inhibitor, DAPT and L685,458. DAPT had the strong effect for 30R which was a relatively sensitive line to MRK003 (supplementary Fig. 4). On the other hand, L685,458 reduced sphere formation not only in 30R but also in TGS01 and TGS04 (data not shown). Generally, γ-secretase inhibitors can be classified according to the chemical structures and pharmacological modes of action. MRK003, DAPT and L685,458 are a sulfonamide-containing non-transition state analog, an azepine-containing non-transition state analog and an azepine-containing transition state analog, respectively [42, 49]. The different experimental results with three γ-secretase inhibitors might be caused by different pharmacological modes of action. These inhibitors might modulate different downstream signaling pathways. In fact, these inhibitors affect various intracellular signaling including PI3K/Akt signaling [50].

A further point of investigation in our study was to confirm the role of Notch pathway inhibition in effecting

the MRK003 response, since the possibility of MRK003 acting via off-targets cannot be ruled out. However, accumulated evidence confirmed the strong effect of MRK003 for cancer initiating cells including leukemia [22, 27], lymphoma [25], breast [24, 26], and pancreas [23] in vitro and in vivo. In this study also, MRK003 provided a therapeutic advantage against the chemo resistant population of GICs derived from the nine patients. Regardless of the specificity of MRK003, it promises to be an effective target therapy for GBM.

In conclusion, results of this study suggested that MRK003 might have significant therapeutic potential for CD44-high and CD133-low expressed GICs (supplementary Fig. 5). However, additional pre-clinical studies will be required to address whether MRK003 contributes beneficially to GBM treatment.

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Conflict of interest All authors disclosed no potential conflicts of interest.

References

1. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987–996. doi:10.1056/NEJMoa043330
2. Wen PY, Kesari S (2008) Malignant gliomas in adults. N Engl J Med 359:492–507. doi:10.1056/NEJMra0708126
3. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P (2007) The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 114:97–109. doi:10.1007/s00401-007-0243-4
4. Gilbert MR, Wang M, Aldape KD, Stupp R, Hegi ME, Jaeckle KA, Armstrong TS, Wefel JS, Won M, Blumenthal DT, Mahajan A, Schultz CJ, Erridge S, Baumert B, Hopkins KI, Tzuk-Shina T, Brown PD, Chakravarti A, Curran WJ Jr, Mehta MP (2013) Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. J Clin Oncol 31:4085–4091. doi:10.1200/JCO.2013.49.6968
5. Chen R, Nishimura MC, Bumbaca SM, Kharbanda S, Forrest WF, Kasman IM, Greve JM, Soriano RH, Gilmour LL, Rivers CS, Modrusan Z, Nacu S, Guerrero S, Edgar KA, Wallin JJ, Lamszus K, Westphal M, Heim S, James CD, VandenBerg SR, Costello JF, Moorefield S, Cowdrey CJ, Prados M, Phillips HS (2010) A hierarchy of self-renewing tumor-initiating cell types in glioblastoma. Cancer Cell 17:362–375. doi:10.1016/j.ccr.2009.12.049
6. Tamase A, Muraguchi T, Naka K, Tanaka S, Kinoshita M, Hoshii T, Ohmura M, Shugo H, Ooshio T, Nakada M, Sawamoto K, Onodera M, Matsumoto K, Oshima M, Asano M, Saya H, Okano H, Suda T, Hamada J, Hirao A (2009) Identification of tumor-initiating cells in a highly aggressive brain tumor using promoter activity of nucleostemin. Proc Natl Acad Sci USA 106:17163–17168. doi:10.1073/pnas.0905016106
7. Huang Z, Cheng L, Guryanova OA, Wu Q, Bao S (2010) Cancer stem cells in glioblastoma–molecular signaling and therapeutic targeting. Protein Cell 1:638–655. doi:10.1007/s13238-010-0078-y
8. Natsume A, Kinjo S, Yuki K, Kato T, Ohno M, Motomura K, Iwami K, Wakabayashi T (2011) Glioma-initiating cells and molecular pathology: implications for therapy. Brain Tumor Pathol 28:1–12. doi:10.1007/s10014-010-0011-3
9. Ables JL, Breunig JJ, Eisch AJ, Rakic P (2011) Not(ch) just development: notch signalling in the adult brain. Nat Rev Neurosci 12:269–283. doi:10.1038/nrn3024
10. Ranganathan P, Weaver KL, Capobianco AJ (2011) Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 11:338–351. doi:10.1038/nrc3035
11. Kamstrup MR, Gjerdrum LM, Biskup E, Lauenborg BT, Ralfkiaer E, Woetmann A, Odum N, Gniadecki R (2010) Notch1 as a potential therapeutic target in cutaneous T-cell lymphoma. Blood 116:2504–2512. doi:10.1182/blood-2009-12-260216
12. Hu YY, Zheng MH, Cheng G, Li L, Liang L, Gao F, Wei YN, Fu LA, Han H (2011) Notch signaling contributes to the maintenance of both normal neural stem cells and patient-derived glioma stem cells. BMC Cancer 11:82. doi:10.1186/1471-2407-11-82
13. Xu P, Qiu M, Zhang Z, Kang C, Jiang R, Jia Z, Wang G, Jiang H, Pu P (2010) The oncogenic roles of Notch1 in astrocytic gliomas in vitro and in vivo. J Neurooncol 97:41–51. doi:10.1007/s11060-009-0007-1
14. Zhang XP, Zheng G, Zou L, Liu HL, Hou LH, Zhou P, Yin DD, Zheng QJ, Liang L, Zhang SZ, Feng L, Yao LB, Yang AG, Han H, Chen JY (2008) Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. Mol Cell Biochem 307:101–108. doi:10.1007/s11010-007-9589-0
15. Kanamori M, Kawaguchi T, Nigro JM, Feuerstein BG, Berger MS, Miele L, Pieper RO (2007) Contribution of Notch signaling activation to human glioblastoma multiforme. J Neurosurg 106:417–427. doi:10.3171/jns.2007.106.3.417
16. Cancer Genome Atlas Research N (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455:1061–1068. doi:10.1038/nature07385
17. Stockhausen MT, Kristoffersen K, Poulsen HS (2010) The functional role of Notch signaling in human gliomas. Neuro Oncol 12:199–211. doi:10.1093/neuonc/nop022
18. Ulasov IV, Nandi S, Dey M, Sonabend AM, Lesniak MS (2011) Inhibition of Sonic hedgehog and Notch pathways enhances sensitivity of CD133(+) glioma stem cells to temozolomide therapy. Mol Med 17:103–112. doi:10.2119/molmed.2010.00062
19. Chen J, Kesari S, Rooney C, Strack PR, Chen J, Shen H, Wu L, Griffin JD (2010) Inhibition of notch signaling blocks growth of glioblastoma cell lines and tumor neurospheres. Genes Cancer 1:822–835. doi:10.1177/1947601910383564
20. Fan X, Khaki L, Zhu TS, Soules ME, Talsma CE, Gul N, Koh C, Zhang J, Li YM, Maciaczyk J, Nikkhah G, Dimeco F, Piccirillo S, Vescovi AL, Eberhart CG (2010) NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells 28:5–16. doi:10.1002/stem.254

21. Schreck KC, Taylor P, Marchionni L, Gopalakrishnan V, Bar EE, Gaiano N, Eberhart CG (2010) The Notch target Hes1 directly modulates Gli1 expression and Hedgehog signaling: a potential mechanism of therapeutic resistance. Clin Cancer Res 16:6060–6070. doi:10.1158/1078-0432.CCR-10-1624
22. Lewis HD, Leveridge M, Strack PR, Haldon CD, O'Neil J, Kim H, Madin A, Hannam JC, Look AT, Kohl N, Draetta G, Harrison T, Kerby JA, Shearman MS, Beher D (2007) Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. Chem Biol 14:209–219. doi:10.1016/j.chembiol.2006.12.010
23. Mizuma M, Rasheed ZA, Yabuuchi S, Omura N, Campbell NR, de Wilde RF, De Oliveira E, Zhang Q, Puig O, Matsui W, Hidalgo M, Maitra A, Rajeshkumar NV (2012) The gamma secretase inhibitor MRK-003 attenuates pancreatic cancer growth in preclinical models. Mol Cancer Ther 11:1999–2009. doi:10.1158/1535-7163.MCT-12-0017
24. Kondratyev M, Kreso A, Hallett RM, Girgis-Gabardo A, Barcelon ME, Ilieva D, Ware C, Majumder PK, Hassell JA (2012) Gamma-secretase inhibitors target tumor-initiating cells in a mouse model of ERBB2 breast cancer. Oncogene 31:93–103. doi:10.1038/onc.2011.212
25. Ramakrishnan V, Ansell S, Haug J, Grote D, Kimlinger T, Stenson M, Timm M, Wellik L, Halling T, Rajkumar SV, Kumar S (2012) MRK003, a gamma-secretase inhibitor exhibits promising in vitro pre-clinical activity in multiple myeloma and non-Hodgkin's lymphoma. Leukemia 26:340–348. doi:10.1038/leu.2011.192
26. Efferson CL, Winkelmann CT, Ware C, Sullivan T, Giampaoli S, Tammam J, Patel S, Mesiti G, Reilly JF, Gibson RE, Buser C, Yeatman T, Coppola D, Winter C, Clark EA, Draetta GF, Strack PR, Majumder PK (2010) Downregulation of Notch pathway by a gamma-secretase inhibitor attenuates AKT/mammalian target of rapamycin signaling and glucose uptake in an ERBB2 transgenic breast cancer model. Cancer Res 70:2476–2484. doi:10.1158/0008-5472.CAN-09-3114
27. Tammam J, Ware C, Efferson C, O'Neil J, Rao S, Qu X, Gorenstein J, Angagaw M, Kim H, Kenific C, Kunii K, Leach KJ, Nikov G, Zhao J, Dai X, Hardwick J, Scott M, Winter C, Bristow L, Elbi C, Reilly JF, Look T, Draetta G, Van der Ploeg L, Kohl NE, Strack PR, Majumder PK (2009) Down-regulation of the Notch pathway mediated by a gamma-secretase inhibitor induces anti-tumour effects in mouse models of T-cell leukaemia. Br J Pharmacol 158:1183–1195. doi:10.1111/j.1476-5381.2009.00389.x
28. Jin R, Nakada M, Teng L, Furuta T, Sabit H, Hayashi Y, Demuth T, Hirao A, Sato H, Zhao G, Hamada J (2013) Combination therapy using Notch and Akt inhibitors is effective for suppressing invasion but not proliferation in glioma cells. Neurosci Lett 534:316–321. doi:10.1016/j.neulet.2012.12.008
29. Chu Q, Orr BA, Semenkow S, Bar EE, Eberhart CG (2013) Prolonged inhibition of glioblastoma xenograft initiation and clonogenic growth following in vivo notch blockade. Clin Cancer Res 19:3224–3233. doi:10.1158/1078-0432.CCR-12-2119
30. Saito N, Fu J, Zheng S, Yao J, Wang S, Liu DD, Yuan Y, Sulman EP, Lang FF, Colman H, Verhaak RG, Yung WK, Koul D (2014) A high notch pathway activation predicts response to gamma secretase inhibitors in proneural subtype of glioma tumor-initiating cells. Stem Cells 32:301–312. doi:10.1002/stem.1528
31. Gu C, Banasavadi-Siddegowda YK, Joshi K, Nakamura Y, Kurt H, Gupta S, Nakano I (2013) Tumor-specific activation of the C-JUN/MELK pathway regulates glioma stem cell growth in a p53-dependent manner. Stem Cells 31:870–881. doi:10.1002/stem.1322
32. Joshi K, Banasavadi-Siddegowda Y, Mo X, Kim SH, Mao P, Kig C, Nardini D, Sobol RW, Chow LM, Kornblum HI, Waclaw R, Beullens M, Nakano I (2013) MELK-dependent FOXM1 phosphorylation is essential for proliferation of glioma stem cells. Stem Cells 31:1051–1063. doi:10.1002/stem.1358
33. Mao P, Joshi K, Li J, Kim SH, Li P, Santana-Santos L, Luthra S, Chandran UR, Benos PV, Smith L, Wang M, Hu B, Cheng SY, Sobol RW, Nakano I (2013) Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc Natl Acad Sci USA 110:8644–8649. doi:10.1073/pnas.1221478110
34. Miyazaki T, Pan Y, Joshi K, Purohit D, Hu B, Demir H, Mazumder S, Okabe S, Yamori T, Viapiano M, Shin-ya K, Seimiya H, Nakano I (2012) Telomestatin impairs glioma stem cell survival and growth through the disruption of telomeric G-quadruplex and inhibition of the proto-oncogene, c-Myb. Clin Cancer Res 18:1268–1280. doi:10.1158/1078-0432.CCR-11-1795
35. Ikushima H, Todo T, Ino Y, Takahashi M, Miyazawa K, Miyazono K (2009) Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. Cell Stem Cell 5:504–514. doi:10.1016/j.stem.2009.08.018
36. Muraguchi T, Tanaka S, Yamada D, Tamase A, Nakada M, Nakamura H, Hoshii T, Ooshio T, Tadokoro Y, Naka K, Ino Y, Todo T, Kuratsu J, Saya H, Hamada J, Hirao A (2011) NKX2.2 suppresses self-renewal of glioma-initiating cells. Cancer Res 71:1135–1145. doi:10.1158/0008-5472.CAN-10-2304
37. Yamada D, Hoshii T, Tanaka S, Hegazy AM, Kobayashi M, Tadokoro Y, Ohta K, Ueno M, Ali MA, Hirao A (2014) Loss of Tsc1 accelerates malignant gliomagenesis when combined with oncogenic signals. J Biochem. doi:10.1093/jb/mvt112
38. Yan X, Ma L, Yi D, Yoon JG, Diercks A, Foltz G, Price ND, Hood LE, Tian Q (2011) A CD133-related gene expression signature identifies an aggressive glioblastoma subtype with excessive mutations. Proc Natl Acad Sci USA 108:1591–1596. doi:10.1073/pnas.1018696108
39. Lottaz C, Beier D, Meyer K, Kumar P, Hermann A, Schwarz J, Junker M, Oefner PJ, Bogdahn U, Wischhusen J, Spang R, Storch A, Beier CP (2010) Transcriptional profiles of CD133+ and CD133− glioblastoma-derived cancer stem cell lines suggest different cells of origin. Cancer Res 70:2030–2040. doi:10.1158/0008-5472.CAN-09-1707
40. Jijiwa M, Demir H, Gupta S, Leung C, Joshi K, Orozco N, Huang T, Yildiz VO, Shibahara I, de Jesus JA, Yong WH, Mischel PS, Fernandez S, Kornblum HI, Nakano I (2011) CD44v6 regulates growth of brain tumor stem cells partially through the AKT-mediated pathway. PLoS ONE 6:e24217. doi:10.1371/journal.pone.0024217
41. Hurlbut GD, Kankel MW, Lake RJ, Artavanis-Tsakonas S (2007) Crossing paths with notch in the hyper-network. Curr Opin Cell Biol 19:166–175. doi:10.1016/j.ceb.2007.02.012
42. Olsauskas-Kuprys R, Zlobin A, Osipo C (2013) Gamma secretase inhibitors of notch signaling. OncoTargets Ther 6:943–955. doi:10.2147/ott.s33766
43. Anido J, Saez-Borderias A, Gonzalez-Junca A, Rodon L, Folch G, Carmona MA, Prieto-Sanchez RM, Barba I, Martinez-Saez E, Prudkin L, Cuartas I, Raventos C, Martinez-Ricarte F, Poca MA, Garcia-Dorado D, Lahn MM, Yingling JM, Rodon J, Sahuquillo J, Baselga J, Seoane J (2010) TGF-beta receptor inhibitors target the CD44(high)/Id1(high) glioma-initiating cell population in human glioblastoma. Cancer Cell 18:655–668. doi:10.1016/j.ccr.2010.10.023
44. Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, Jeon JW, Kim MH, Kang BG, Jung Y, Jin J, Hong SC, Park WY, Lee DS, Kim H, Nam DH (2008) Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Investig 88:808–815. doi:10.1038/labinvest.2008.57
45. Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL, Yu JS (2006) Analysis of gene expression and

chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer 5:67. doi:10.1186/1476-4598-5-67

46. Joseph JV, Balasubramaniyan V, Walenkamp A, Kruyt FA (2013) TGF-beta as a therapeutic target in high grade gliomas—promises and challenges. Biochem Pharmacol 85:478–485. doi:10.1016/j.bcp.2012.11.005
47. Sjolund J, Bostrom AK, Lindgren D, Manna S, Moustakas A, Ljungberg B, Johansson M, Fredlund E, Axelson H (2011) The notch and TGF-beta signaling pathways contribute to the aggressiveness of clear cell renal cell carcinoma. PLoS ONE 6:e23057. doi:10.1371/journal.pone.0023057
48. Bhat KP, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, Wani K, Heathcock L, James JD, Goodman LD, Conroy S, Long L, Lelic N, Wang S, Gumin J, Raj D, Kodama Y, Raghunathan A, Olar A, Joshi K, Pelloski CE, Heimberger A, Kim SH, Cahill DP, Rao G, Den Dunnen WF, Boddeke HW, Phillips HS, Nakano I, Lang FF, Colman H, Sulman EP, Aldape K (2013) Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. Cancer Cell 24:331–346. doi:10.1016/j.ccr.2013.08.001
49. Takebe N, Nguyen D, Yang SX (2014) Targeting notch signaling pathway in cancer: clinical development advances and challenges. Pharmacol Ther 141:140–149. doi:10.1016/j.pharmthera.2013.09.005
50. Wang J, Wakeman TP, Lathia JD, Hjelmeland AB, Wang XF, White RR, Rich JN, Sullenger BA (2010) Notch promotes radioresistance of glioma stem cells. Stem Cells 28:17–28. doi:10.1002/stem.261

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ORIGINAL ARTICLE

Detection of *APC* mosaicism by next-generation sequencing in an FAP patient

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Familial adenomatous polyposis (FAP) of the colon is characterized by multiple polyps in the intestine and extra-colonic manifestations. Most FAP cases are caused by a germline mutation in the tumor-suppressor gene *APC*, but some cases of adenomatous polyposis result from germline mutations in *MUTYH*, *POLD1* or *POLE*. Although sequence analysis of *APC* by the Sanger method is routinely performed for genetic testing, there remain cases whose mutations are not detected by the analysis. Next-generation sequencing has enabled us to analyze the comprehensive human genome, improving the chance of identifying disease causative variants. In this study, we conducted whole-genome sequencing of a sporadic FAP patient in which we did not find any pathogenic *APC* mutations by the conventional Sanger sequencing. Whole-genome sequencing and subsequent deep sequencing identified a mosaic mutation of c.3175G>T, p.E1059X in ~12% of his peripheral leukocytes. Additional deep sequencing of his buccal mucosa, hair follicles, non-cancerous mucosa of the stomach and colon disclosed that these tissues harbored the *APC* mutation at different frequencies. Our data implied that genetic analysis by next-generation sequencing is an effective strategy to identify genetic mosaicism in hereditary diseases.
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INTRODUCTION

Approximately 5% of colorectal cancer is caused by hereditary tumor syndromes including Lynch syndrome and familial polyposis of the colon. Their diagnosis is crucial not only for the treatment of patients, but also the healthcare of their relatives. Genetic testing is, therefore, of great importance for the diagnosis and the identification of the mutation carriers in the relatives.[1] Familial adenomatous polyposis (FAP) is an autosomal-dominant colorectal cancer predisposition syndrome that accounts for ~1% of newly diagnosed colorectal cancer cases. FAP is characterized by the development of multiple adenomatous polyps ranging from hundreds to thousands in the large intestine. A majority of the patients carry a germline mutation in the tumor-suppressor gene *APC* (adenomatous polyposis coli), but a small number of adenomatous polyposis cases are caused by germline mutations in *MUTYH*, *POLD1* or *POLE*. Genetic tests are performed for the screening of *APC*, but pathogenic mutations are not detected in some FAP cases by conventional direct sequencing owing to several reasons; (i) the region of testing is often restricted to 5′-half in the coding region and does not usually include the 3′-half, promoter region, or the 5′- or 3′-UTR. (ii) Structural alteration comprising large deletions/insertions and inversions are hard to identify by the conventional sequencing method and other testing methods are required. (iii) Some cases of adenomatous polyposis are caused by mutations in other genes such as *MUTYH*, *POLD1* and *POLE*.[2,3] (iv) Some FAP cases are caused by somatic mosaicism of *APC*.[4–7]

Large-scale genome sequencing, also known as next-generation sequencing (NGS), is applicable to germline genomic sequencing, as well as other purposes including sequencing of tumors, sequencing of mRNA to analyze gene expression (RNA-seq), sequencing of DNA enriched by chromatin immunoprecipitation to characterize elements in protein-DNA interactions (ChIP-seq) and others. The entire genome of an individual can be sequenced in less than 1 week for 5000 to 10 000 dollars.[8] Cost reduction, together with advanced bioinformatics capabilities, have led to increased opportunities for NGS usage in various clinical applications including the detection of rare hereditary mutations, individualized therapy, pharmacogenomics, preconception/prenatal screening and population screening for disease risk.[9,10]

Here we show that genetic testing by NGS facilitates to identify mosaic *APC* mutations in patients with attenuated polyposis. NGS will improve genetic diagnosis of hereditary diseases whose mutations have been overlooked by conventional direct sequencing.

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MATERIALS AND METHODS

Patients and ethical approval

This project was approved by the ethical committee of Institute of Medical Science, the University of Tokyo (IMSUT-IRB, 23-18-0929 and 23-19-0929). Written informed consent was obtained from the patient in this study.

Genetic testing

Genomic DNA was extracted from peripheral blood of the patient according to the standard phenol extraction/purification procedure. *APC* coding exons were amplified with M13-tailed target specific primers, and the PCR products were sequenced on the Applied Biosystems 3730×l DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) using the BigDye Direct Cycle Sequencing Kit (Thermo Fisher Scientific). The primer sequences used for sequencing are available on request.

Whole-genome sequencing

We prepared insert libraries of 250–350 bp from 1.0 μg of genomic DNA from lymphocytes and sequenced them using the HiSeq 2000 platforms with paired-end reads of 101 bp according to the manufacturer's instructions (Illumina, San Diego, CA, USA). For the data processing, fastq files were aligned to human reference sequence (hg19) by BWA[11] (ver. 0.5.10) and a bam file was created. For the detection of variants, we compared the bam file with the reference sequence by a Bayesian approach. We used a beta non-informative prior for representing the probability of existence of variant and the posterior distribution was obtained using the information of observed reads at each candidate position. We supposed that a random variable 'X' follows the posterior distribution representing the probability of variant. We used $Pr(X \geqslant 0.05)$ as the score, and regarded the candidate positions whose scores were greater than 0.9 as statistically significant.

Validation of variants detected by NGS

To confirm the mutation, DNA of peripheral blood was amplified independently by PCR (KOD-Plus kit, TOYOBO, Osaka, Japan) with a set of primers encompassing the possible pathogenic mutation, and direct sequence was performed by the Sanger method. The primer sequences used for amplification are shown in Supplementary Table 1. To confirm mosaicism, genomic DNA was extracted from hair follicles, two sites of buccal mucosa, three sites of non-tumorous stomach mucosa, five sites of non-tumorous colonic mucosa and five adenomatous polyps from the patient by the standard phenol extraction/purification procedure. Deep sequencing was carried out using IonPGM Sequencing 200 kit and Sequencing 400 kit (Thermo Fisher Scientific) with libraries of PCR products prepared using Ion Plus Fragment Library Kit (Thermo Fisher Scientific). Variants were identified using the Variant Caller deployed with Torrent Suite (Thermo Fisher Scientific).

RESULTS

In clinical genetic testing, we encountered a male, 41 years of age, who suffered from multiple polyps in his large intestine. He earlier visited a hospital for the secondary screening of his intestine because of occult blood in his fecal test. Colonoscopy detected <100 adenomatous polyps in his large intestine and subsequent histological examination of the polyps diagnosed adenomatosis. As he had no family history of polyposis or colorectal cancer, he was suspected to be a *de novo* case of FAP or a patient of MUTYH-associated polyposis. Direct sequencing of *APC* was performed using DNA extracted from his lymphocytes to examine the 5′-half of the coding region where most of the *APC* mutations occur; however, no pathogenic mutations were detected. Although structural analysis of *APC* by Multiplex Ligation-dependent Probe Amplification, or sequencing of *MUTYH*, *POLD* and *POLE* by the Sanger method were available for the second screening, we performed whole-genome sequencing to test the usefulness of NGS in clinical testing.

The average depth of sequence coverage was ~26x, and a total of 4.6 million variants were identified in the patient. Because three to four million variants are generally detected by whole-genome sequencing in an individual,[9] we considered that the number of variants was reasonable. Among the 4.6 million, 30 501 variants were located in the exonic regions, and 8 were detected in the exons or splicing regions of *APC* (Table 1). Importantly, one of the eight variants was a nonsense mutation (c.3175G>T p.E1059X) that was determined in 6 of 50 reads (12%). Although the frequency was much lower than 50%, we suspected that it may be the causative mutation of his polyposis because the nonsense mutation truncates the APC protein leading to the loss of domains involved in β-catenin degradation. We re-sequenced the region by the Sanger method, and found a low peak of mutant allele compared with the wild allele in his DNA from peripheral blood. Taken together with his family history and the number of polyps, we suspected the possibility of *APC* mosaicism. The Sanger method is apparently less sensitive for the detection of mosaicism, compared with NGS (Figure 1). Notably, NGS identified no deleterious mutation in *MUTYH*, *POLD1* or *POLE* (data not shown). The remaining seven *APC* variants had been detected in the initial screening.

We further performed a deep sequencing of his DNA isolated form peripheral blood, hair follicles and two sites of oral mucosa. The average depth of coverage achieved with amplicon sequencing was 34 699x ranging from 3726 to 87 133. As shown in Table 2, c.3175G>T mutation was observed in 453 of 3726 reads (12.2%) in peripheral blood, 3774 of 83 679 (4.5%) in hair follicles, and 2099 of 69 169 (3.0%) and 4860 of 66 557 (7.3%) in buccal mucosa. Because the patient underwent gastric endoscopy and total colectomy, we also examined the mutation in three spots of non-tumorous gastric mucosa, five spots of non-tumorous colonic mucosa and five adenomatous polyps in the colon. Interestingly, we found different frequencies of c.3175G>T mutation in non-tumorous gastric (18.9, 22.7 and 27.7%) and colonic (9.2, 3.4, 12.3, 5.8 and 9.0%) tissues (Table 2). In addition, the mutation was found at higher frequencies (32.3, 28.6, 29.8, 32.5 and 24.7%) in the colonic polyps

Table 1 Summary of variations in all *APC* exons detected by WGS

Type	*Mutation*	*Protein alteration*	*dbSNP131*	*Ref (No of reads)*	*Obs (No of reads)*	*Mismatch ratio*
Synonymous	c.T1458C	p.Y486Y	rs2229992	T (0)	C (75)	1
Synonymous	c.G1635A	p.A545A	rs351771	G (0)	A (35)	1
Nonsense	c.G3175T	p.E1059X	—	G (44)	T (6)	0.12
Synonymous	c.G4479A	p.T1493T	rs41115	G (0)	A (55)	1
Synonymous	c.G5034A	p.G1678G	rs42427	G (0)	A (67)	1
Synonymous	c.T5268G	p.S1756S	rs866006	T (0)	G (43)	1
Non synonymous	c.T5465A	p.V1822D	rs459552	T (0)	A (39)	1
Synonymous	c.G5880A	p.P1960P	rs465899	G (0)	A (23)	1

Abbreviations: APC, adenomatous polyposis coli; No, number; Obs, observed nucleotide; Ref, reference nucleotide; WGS, whole-genome sequencing.

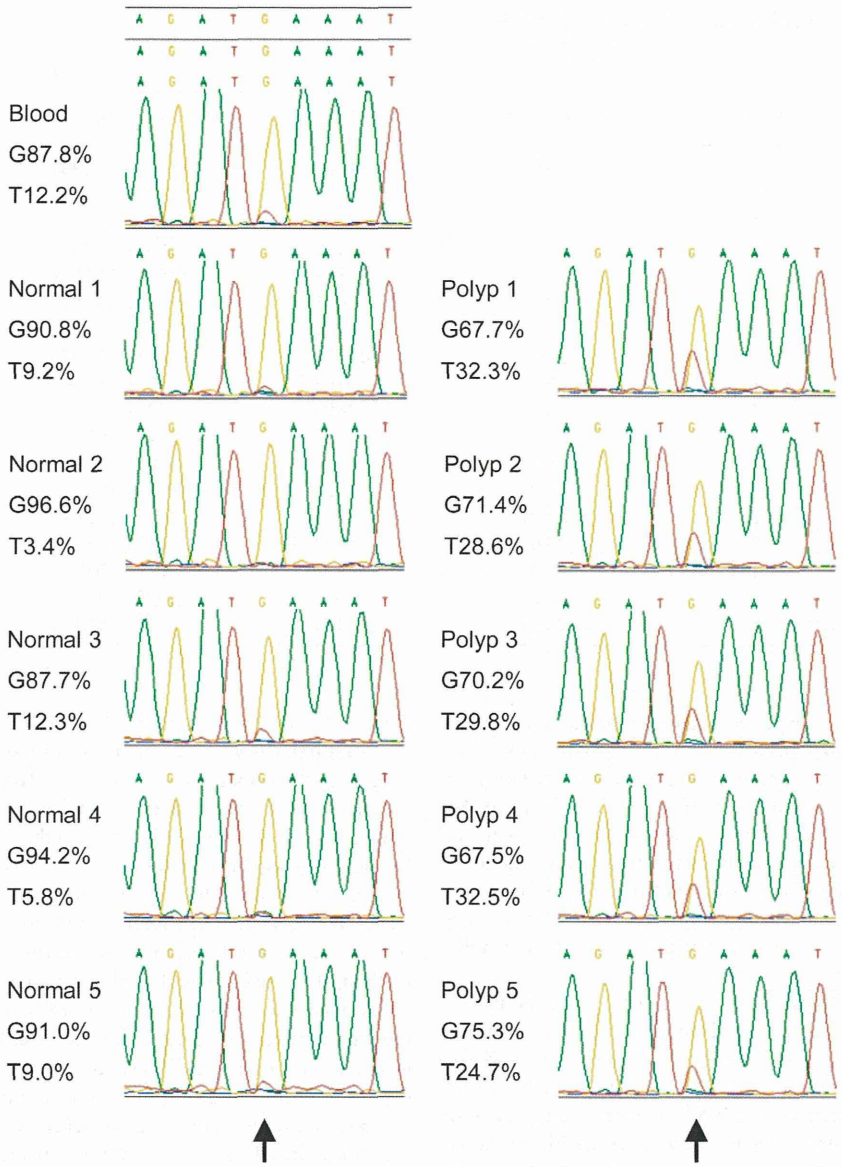

Figure 1 Direct sequencing of the mutant allele (c.3175G>T) in peripheral blood, non-cancerous colonic mucosae and adenomas of the colon. The arrows indicate the position of mutation. The corresponding results obtained from deep sequencing are shown on the left.

than the non-tumorous mucosa (Table 2). These data implied that the c.3175G>T mutation has a crucial role in the development of adenoma, and are consistent with the view of a clonal expansion of cells carrying *APC* mutation. As the average frequency of *APC* mutation in the polyps is ~30%, we can estimate that between 50 and 60% of the polyps are composed of tumor cells if loss of heterozygosity is not involved in the tumorigenesis.

DISCUSSION

In this report, we have shown that NGS is a powerful tool to identify mosaicism of *APC* mutation in a patient with polyposis. Although reports of somatic *APC* mosaicism are limited, recent studies have demonstrated that the mosaicism (10–20%) is present in a significant number of FAP patients harboring *de novo* germline mutation of *APC*.[4,6] Therefore, *APC* mosaicism may have been underestimated compared with other tumor-suppressor genes such as *RB1*, *TSC1* and *TSC2*.[12–14]

According to the number of polyps and age of onset, FAP is divided into three clinical subtypes: attenuated (mild), classical (typical) and severe.[15,16] A genotype–phenotype correlation has been well known in FAP. A recent study confirmed that patients with *APC* mutations between codon 1249–1549 certainly developed polyposis at an early age and exhibited a worse survival. On the other hand, patients with *APC* mutations in codon <178 or 312–412 have a later onset of polyposis and exhibited an improved survival.[17] Aretz *et al.*[4] previously reported eight cases with *APC* mosaic mutations. Although the eight mutations were located between codons 216–1464 associated with classical or severe phenotype, most of the patients exhibited mild phenotype. As the cells carrying *APC* mutation are scattered at a lower frequency in the epithelia of colonic mucosa compared with the classical FAP, the patients with mosaic mutations likely have a milder