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Original Article

The Establishment of a Fast and Safe Orthotopic Colon Cancer Model Using a Tissue Adhesive Technique

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Purpose We aimed to develop a novel method for orthotopic colon cancer model, using tissue adhesive in place of conventional surgical method.

Materials and Methods RFP HCT 116 cell line were used to establish the colon cancer model. Fresh tumor tissue harvested from a subcutaneous injection was grafted into twenty nude mice, divided into group A (suture method) and group B (tissue adhesive method). For the group A, we fixed the tissue on the serosa layer of proximal colon by 8-0 surgical suture. For the group B, tissue adhesive (10 μL) was used to fix the tumor. The mortality, tumor implantation success, tumor metastasis, primary tumor size, and operation time were compared between the two groups. Dissected tumor tissue was analyzed for the histology and immunohistochemistry. Also, we performed tumor marker analysis.

Results We observed 30% increase in graft success and 20% decrease in mortality, by using tissue adhesive method, respectively. The median colon tumor size was significantly increased by 4 mm and operation time was shortened by 6.5 minutes. The H&E showed similar tumor structure between the two groups. The immunohistochemistry staining for cancer antigen 19-9, carcinoembryonic antigen, cytokeratin 20, and Ki-67 showed comparable intensities in both groups. Real-time quantitative reverse transcription analysis showed eight out of nine tumor markers are unchanged in the tissue adhesive group. Western blot indicated the tissue adhesive group expressed less p-JNK (apototic marker) and more p-MEK/p-p38 (proliferation marker) levels.

Conclusion We concluded the tissue adhesive method is a quick and safe way to generate orthotopic, colon cancer model.

Key words Orthotopic, Colon neoplasms, Mouse model, Tissue adhesive technique

Introduction

Colorectal cancer has become a major disease threatening human health in the world [1]. Establishing the suitable animal models of human colorectal cancer will not only help us to explore the development mechanisms of colorectal cancer effectively but also provide the chance for the examination of the therapeutic candidates [2]. According to our knowledge, commonly used animal models for colorectal cancer include an orthotopic transplantation model, subcutaneous tumor model, gene knock-out model, and the chemically induced model [3]. But each of them has certain limitations. For better keeping the biological properties of human colorectal cancer, the tumor microenvironment in human should be created similarly in animal model. Ideal model can keep the interaction between tumor cells or tumor cells and stroma. In this regard, the orthotopic tumor model can mimic the natural environment of the original tumor more accurately. Thus, it provides an effective tool to investigate tumor pathophysiology and develop therapeutic strategies [3].

It has been reported that the orthotopic tumor cell injection or tumor tissue transplantation can mimic spontaneous tumor growth and metastasis [4,5]. However, most of the cell line for injection use commercial cell lines that have been cultured for many years. Furthermore, the rate of metastasis caused by the orthotopic injection of cell suspension appears to be minor, compared to orthotopic implantation of intact tumor tissue. Therefore, the use of tumor tissue for the ortho-

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topic transplantation can more accurately reflects the clinical features of human colon cancer in nude mice [4]. The commonly used method for the tumor tissue graft is to implant it subcutaneously first, and after the tumor grows up, re-graft the tumor for the orthotopic xenograft [4].

The conventional transplantation technique for developing orthotopic colon cancer involves a microsurgical technique, suturing an intact colon cancer tissue onto the cecal wall of nude mice. This technique is challenging and requires specialized equipment, such as 8-0 fine suture and surgical microscope as well as a good surgical skill [6]. The technique includes exposing the cecum or colon, scratching the serosa and then fixing a tumor piece on top, by suturing it onto the wall. During this procedure, tumor tissue and normal colon wall must be sutured carefully not to injure the colon tissue, otherwise often result in bleeding and perforation [6]. As an alternative of the suturing technique, Kageyama et al. [7] reported a hepatic metastasis mouse model by the closure of the incision site with absorbable hemostatic materials.

In this study, we reasoned that the use of tissue adhesive would be a simpler and safer method to establish an orthotopic colon cancer mouse model, compared to the traditional suturing method. By comparing the differences between two methods in tumor growth, pathological manifestations and colon biomarker expressions, we validated the usefulness of the new method.

Materials and Methods

1. Cell culture

The HCT 116 humanity colon cancer cell line were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin. Cells were grown at 37°C and 5% CO_2 in an incubator.

2. Animals and study design and tumor tissue preparation

Six-week-old male nude mice were used in this study. All animals fed with autoclaved laboratory rodent diet and were housed in individually ventilated cages at 24°C with a controlled 12-hour day-night cycle. Stocks of HCT 116 tumors were established by subcutaneously injecting HCT 116 cells (5×10^6) in the right flank of two nude mice. Colon tumors were harvested at the exponential growth phase and resected under aseptic conditions. Necrotic tissues were removed and viable tissues were cut with scissors and minced into 1 mm^3 piece to be prepared for the implantation. A total of 20 nude mice were divided into group A and group B with each 10 mice. The mice of group A received orthotopic implantation by surgical suture method and the mice of group B received the surgical tissue adhesive method.

3. Orthotopic implantation using the suture method

The suture method was performed described previously [8]. Briefly, the mice were anesthetized with subcutaneous injection of a mixture of 50 mg/kg tiletamine (Zoletil 50, Virbac, Carros, France) and 10 mg/kg xylazine (Rompun, Bayer HealthCare, Leverkusen, Germany). And then the mouse was placed on a heating pad in the supine position. After the sterilization of the surgical area, a midline incision in the lower abdomen was made, and then the proximal colon was dissected and exposed. The serosa of the proximal colon was scratched with a blade and one 1 mm^3 tumor fragments per mouse were implanted on the wall of the proximal colon with an 8-0 surgical suture (Fig. 1A-D). The proximal colon was then returned to the peritoneal cavity. The muscle layer was closed by 5-0 absorbable sutures, and the skin wound was closed by 5-0 non-absorbable sutures. All procedures of the operation described above were performed under an 8 x magnification microscope. We record the suture method procedure time and it is defined as from opening the abdomen to the end of the skin wound closing.

4. Orthotopic implantation using the tissue adhesive method

After exposure and scratched the proximal colon serosa with a blade, one 1 mm^3 tumor fragments per mouse were attached to the damaged area of the colon serosa. A gauze with a hole was covered in the colon to protect the near tissue and then then 10 μL tissue adhesive (cyanoacrylate, B. Braun, Melsungen, Germany) was dropped to cover the tumor pieces (Fig. 1E-H). After this, additional 1 minute was given to make sure the adhesive solidification and to avoid the adhesion onto the surrounding tissues. The proximal colon was then returned to the peritoneal cavity and incision in the abdominal wall was closed. The muscle layer was closed by 5-0 absorbable sutures, and the skin wound was closed by 5-0 non-absorbable sutures. Mice were then allowed to rest on a heating pad until full recovery. We measured the duration of tissue adhesive method and suture method for comparison.

5. Tumor sample collection and tumor size measurement

Tumor implantation success rate is defined as after the surgical implantation, the mouse lives well at least 1 week. Tumor formation is defined as tumor growth that can be seen while sacrificing. The tumor samples were collected from the mice 8 weeks after orthotopic implantation. The tumor size of each sample was measured with a caliper and the max diameter was recorded. After washed with sterile PBS, the tumor samples were divided into three pieces and used for reverse transcription polymerase chain reaction, Western blot, histology and immunohistochemistry analysis.

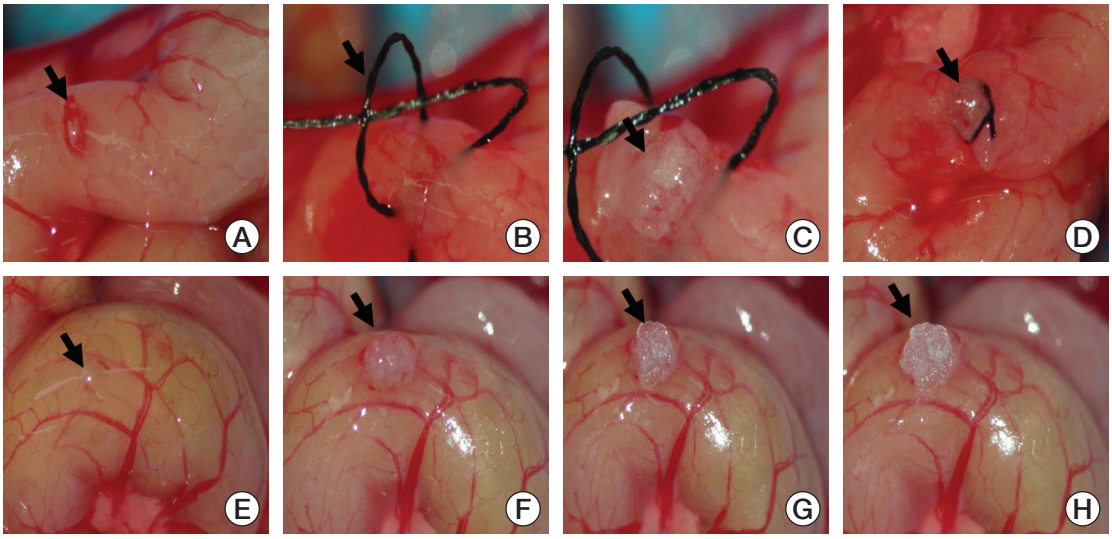

Fig. 1. The development of tissue adhesive method for orthotopic tumor implantation on mouse colon. (A) A representative pictures of the proximal colon with small incision to expose serosa. (B) Suturing prepared before the tumor tissue attachment. (C) One 1 mm^3 tumor fragment was implanted on the damaged colon wall of the proximal colon. (D) Stitch the tissue on the colon wall with an 8-0 surgical suture. (E) The serosa of the proximal colon was exposed by small incision. (F) One 1 mm^3 tumor fragment was attached on the damaged colon wall. (G) One small drop of tissue adhesive was applied to cover the tumor piece. The image was captured 10 seconds after application. (H) A picture of fixed tumor on colon after 60 seconds, showing the tissue adhesive was fully solidified (A-H, ×8). Arrows indicate the sites of incision and tumor placement, by suture or tissue adhesive glue.

6. Histology and immunohistochemistry

For histopathological evaluation, hematoxylin and eosin (H&E) staining was performed on paraffin-embedded tumor tissue sections. For immunohistochemistry, sections were stained with primary antibodies overnight at 4°C. On the next day; sections were incubated for 30 minutes in ImmPRESS AP Reagent (Vector Laboratories, Burlingame, CA), followed by incubating for 2-15 minutes in ImmPACT NOVA-RED (Vector Laboratories). The following primary antibodies were purchased from Agilent (Santa Clara, CA): cancer antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), cytokeratin 20 (CK20), and Ki-67 [9]. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, In Situ Cell Death Detection Kit (Roche, Penzberg, Germany) was used, followed by the manufacturer's instruction. Ki-67 positive area or TUNEL positivity in immunohistochemistry (IHC) image was measured by ImageJ software (National Institutes of Health, Bethesda, MD).

7. Tumor marker analysis

1) Real-time quantitative reverse transcription PCR

To quantify the expression of genes on mRNA level, we use real-time quantitative reverse transcription PCR (qRT-PCR) method. Cell or tissue RNA were extracted by Tri-RNA Reagent (FATRR 001, FAVORGEN, Ping-Tung, Taiwan). Reverse transcription (RT) reactions were performed using PrimerScript RT Reagent Kit (RR037A, TaKaRa, Kyoto, Japan) with 250 ng RNA as template in 10 μL reaction system. Then the expression of each mRNA was quantified by AMPIGENE qPCR Green System (10014-632, ENZO, New York, NY) with specific primers (S1 Table).

2) Western blot

Cell or tissue samples were lysed by RIPA Buffer (R2002, Biosesang, Seongnam, Korea) with 1× protease inhibitor cocktail (04693132001, Roche) and 1× PhosSTOP phosphatase inhibitor (04906837001, Roche). Proteins then were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and then transferred onto the PVDF blotting membrane (10600023, Amersham, Buckinghamshire, UK). HRP-conjugated rabbit secondary antibody (#7074, CST, Danver, MA), Roche mouse secondary antibody (#7076, CST), HRP-conjugated goat secondary antibody (sc-2354, Santa Cruz Biotechnology, Santa Cruz, CA) and ECL Western Blotting Detection Reagents (16888355, Amersham) were used to examine the signals. ImageJ was used to quantitatively analyze the expression of proteins.

3) Antibodies

Cyclooxygenase-2 (COX2; #12282, CST), CEA (#236M-96,

Table 1. Comparative analysis of tumor formation, metastasis, tumor size, and procedure duration for the two graft methods

Demographics	Suture	Tissue adhesive	p-value
Total	10	10	
Survival, n (%)	7 (70.0)	9 (90.0)	0.264
Tumor formation (rate), n (%)	6/7 (85.7)	9/9 (100)	0.242
Tumor metastasis, n (%)	6/7 (85.7)	9/9 (100)	0.086
Abdomen	6	9	
Liver	2	0	
Tumor size (mm)			
Range	7-16	8-18	0.031
Median	11	15	
Procedure duration (min)			
Range	9.4-13.2	4.3-5.5	< 0.001
Median	11.05	4.55	

Cell Marque, Rocklin, CA), CA19-9 (ab15146, Abcam, Cambridge, UK), PKM2 (3198S, CST), phospho-p38 mitogen-activated protein kinase (MAPK; 9211S, CST), MEK1/2 (8727S, CST), phosphor-MEK1/2 (9154S, CST), ERK (sc-94, Santa Cruz Biotechnology), phosphor-Erk1/2 (4370S, CST), phospho-JNK1/2 (700031, Invitrogen, Carlsbad, CA), and β-actin (sc-47778, Santa Cruz Biotechnology).

8. Statistical analysis

Comparative analysis was done by t test, chi-square test and Mann-Whitney U test. All statistical analyses were performed by using the SPSS program ver. 21 (IBM Corp., Armonk, NY). All tests were two-sided and $p < 0.05$ were considered statistically significant.

Results

1. Tissue adhesive method is a faster and produces larger tumor than suturing method

The tumor implantation was performed in all mice of the two groups. However, in group A (with suturing method), three mice died after the xenograft. Two mice died due to perforation after 1 day , and the other mouse died by infection 2 days after the procedure. For group B (with tissue adhesive method), one mouse died 3 days after the procedure (due to infection) and the rest nine mice were alive until sacrifice. All of the survived mice (n=16) were sacrificed in 8 weeks after the tumor implantation. Hence, the final graft success rate was 67% in group A (6/9) and 90% in group B (9/9) (Table 1). Abdominal and mesenteric metastasis occurred in six mice of group A (S2 Fig.) and nine mice of group B (S3 Fig.). There has liver metastasis in two mice in group A only and no lung metastasis were observed in both of the two groups.

As the tissue adhesive method does not need suturing, we measured the procedure duration for each method. Our records showed the suturing method took time from 9.4 to 13.2 minutes whereas the tissue adhesive method took 4.3 to 5.5 minutes. The median procedure time was 11 minutes and 4.5 minutes for suturing and tissue adhesive, respectively. We also measured the size of the primary tumor on sacrifice. The results in Table 1 summarizes the size ranging from 7 to 16 mm (median; 11 mm) in suturing and 8 to 18 mm (median, 15 mm) in tissue adhesive method, indicating the latter methods can produce larger tumors. Statistical analysis showed a significant difference in procedure time ($p < 0.01$) and colon tumor size ($p=0.031$) between the two methods. All of the above data were summarized in Table 1.

2. Histological analysis reveals comparable pattern of tumor formation between the two methods

In order to examine the molecular feature of the tumor generated by tissue adhesive, we first performed H&E staining. As the grafted tissue was generated from HCT116 colon cancer cell line, the tumor likely to have homogenous cellularity and structure. Indeed, the images from both suturing and tissue adhesive tumors showed similar cell morphology and structure of tumor margin, which is also found in the liver and mesenteric metastasis (Fig. 2A-F). In some region, we could observe organized structure and high degree of immune cell infiltration, but these patterns were equally seen in both graft groups (Fig. 2G-J).

To further check the histological characters of tumor from tissue adhesive group, we performed IHC staining with biomarkers including CA19-9, CEA, CK20 (tumor markers), Ki-67 (proliferation marker), and TUNEL assay for measuring apoptosis. The result showed a similar degree of inten-

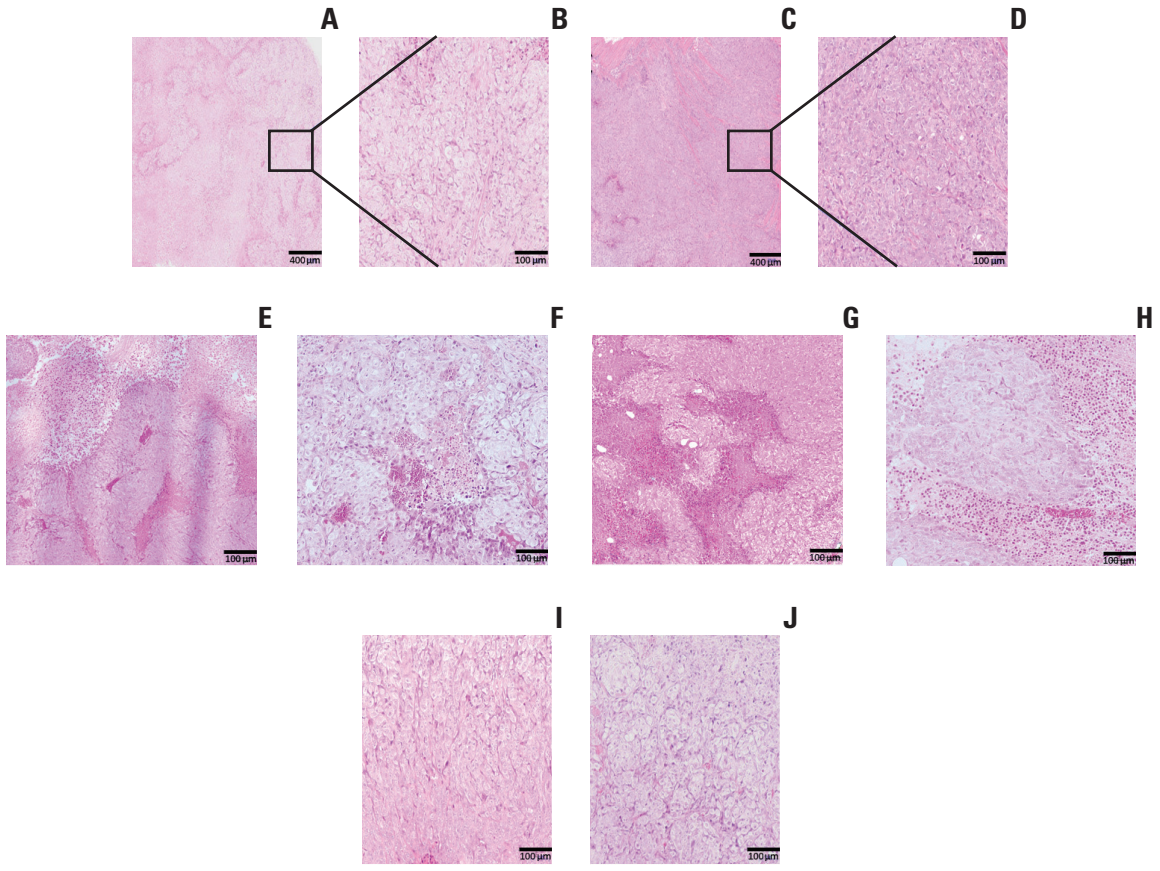

Fig. 2. Tissue adhesive method maintains the gross histology of colon tumors, compared to conventional suture method. (A, B) H&E staining images of the tumor generated by suture method. (C, D) H&E staining images of the tumor generated by the tissue adhesive method. Representative images were taken at ×40 and ×200 magnification. (E, F) Liver metastasis (E) or mesenteric metastasis (F) of colon tumor from gluing method. (G-J) Representative pictures of organized tissue pattern and immune cell infiltration on suture (G, H) and tissue adhesive (I, J) methods. Scale bars=400 μm (A, C), 100 μm (B, D-J).

sity and expression pattern for CA19-9 both in suture and tissue adhesive group (Fig. 3A, top panels). We found stronger signal of CEA and CK20 in suture group but it seems mostly extracellular or non-tumor cells. Quantitative analysis of Ki-67 staining showed comparable portion of Ki-67 positive cells (Fig. 3B, S4 Fig. for raw data). Interestingly, some tumors in the TUNEL assay showed relatively high TUNEL-positive cells (Fig. 3C, raw data in S5 Fig.), but such cases occurred in both of tissue adhesive and suture groups.

3. Molecular analysis of tumors revealed comparable levels of tumor/proliferation marker expression in suturing and tissue adhesive groups

To estimate the validity of tissue adhesive graft method, we further analyzed tumors by qRT-PCR and western blot for known biomarkers of colon cancer, COX2 [10], CEA [11], CA19-9 [12], and cancer stem cell biomarker CD133 [13]. We also included JNK (apoptosis marker) [14], p38 (proliferation and differentiation marker) [15], ERK [16], MEK2 (proliferation and migration marker) [17], IGFBP2 (migration marker) [18], PKM2 (metabolic marker) [19], CDK2 [20], and CCNA2 (cell cycle marker) [21]. We added HCT-116 cell line and HCT-116 derived, subcutaneous tumors as control. The data in Fig. 4 showed most of the tested markers did not show significant expression changes in two groups, indicating tissue adhesive method did not alter molecular nature of the tumor. We found with one exception, though, that tissue adhesive group expressed lower level of CEA on mRNA level. CEA is a well-known colon tumor marker, implicated with metastasis. Thus, this result is in a line with the data in Table 1, showing suture method resulted in more liver metastasis than tissue adhesive method; however, considering the tissue adhesive method also showed evident spreading of the tumors in the mouse abdomen (S3 Fig.), the effect of CEA level change might be functionally marginal.

Given that qRT-PCR cannot exclude various changes in

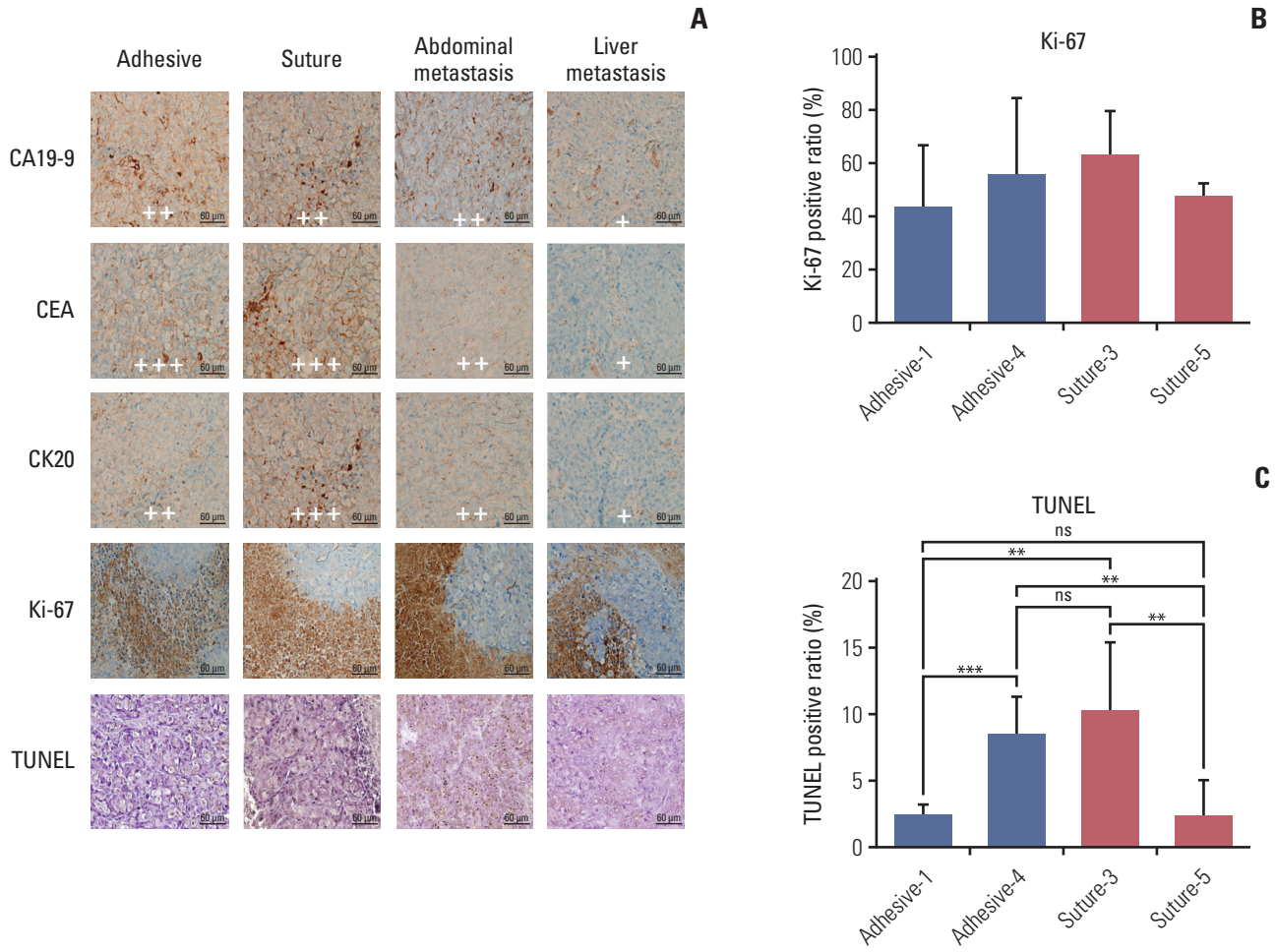

Fig. 3. Immunohistochemical staining for CA19-9, CEA, CK20, and Ki-67 reveals comparable expression pattern between tissue adhesive and suture method. (A) Representative pictures of immunohistochemistry from tumors of tissue adhesive or suturing method. CA-19-1, CEA, CK20, Ki-67, and TUNEL are examined. Tumors obtained from liver metastasis or abdominal metastasis were also included. For CA19-9, CEA, and CK20, the relative intensity is marked as +, ++, and +++. Scale bars=60 μM. (B) Graph showing the Ki-67 positive area of tissue adhesive and suture group (n=4). (C) Graph showing average TUNEL postive cells of tissue adhesive and suture group (n=4). CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; CK20, cytokeratin 20; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling. $^{**}p < 0.01$, $^{***}p < 0.001$; n.s., not significant.

post-transcriptional level, especially phosphorylation of proteins, we further examined tumor marker expression on protein level by western blotting (Fig. 5). We found, in contrast to cell lines, subcutaneous tumor displayed higher expression of CA19-9, p-JNK, p-p38, and p-MEK, implying that these genes may play more roles in subcutaneous tumor progression. In addition, CA19-9 and p-JNK, expressed in both subcutaneous and suture group, were lower in tissue adhesive group, suggesting that graft site and method influences some marker expressions. In contrast, CEA expression showed no significate difference among three different models, demonstrating that CEA is a general biomarker for colon cancer. Also, ERK and p-ERK were remained stable in all three groups, indicating that MEK pathway is already activated well in cell line level. Besides, the expression of p-p38, PKM2, MEK, and p-MEK, were more abundant in the tissue adhesive group than suture group. Overall, even though there are some molecular differences, colon tumors generated by the tissue adhesive method show comparable molecular profile to the tumor from suturing method.

Notably, we found that tumor size was bigger and metastasis rate was lower in tissue adhesive group than suture group. As previous study demonstrated that MAPK signaling plays a vital role in colon cancer development [14], so we measured MAPK signaling activity in two groups. The results in Fig. 4 indicated that the expression of MEK and

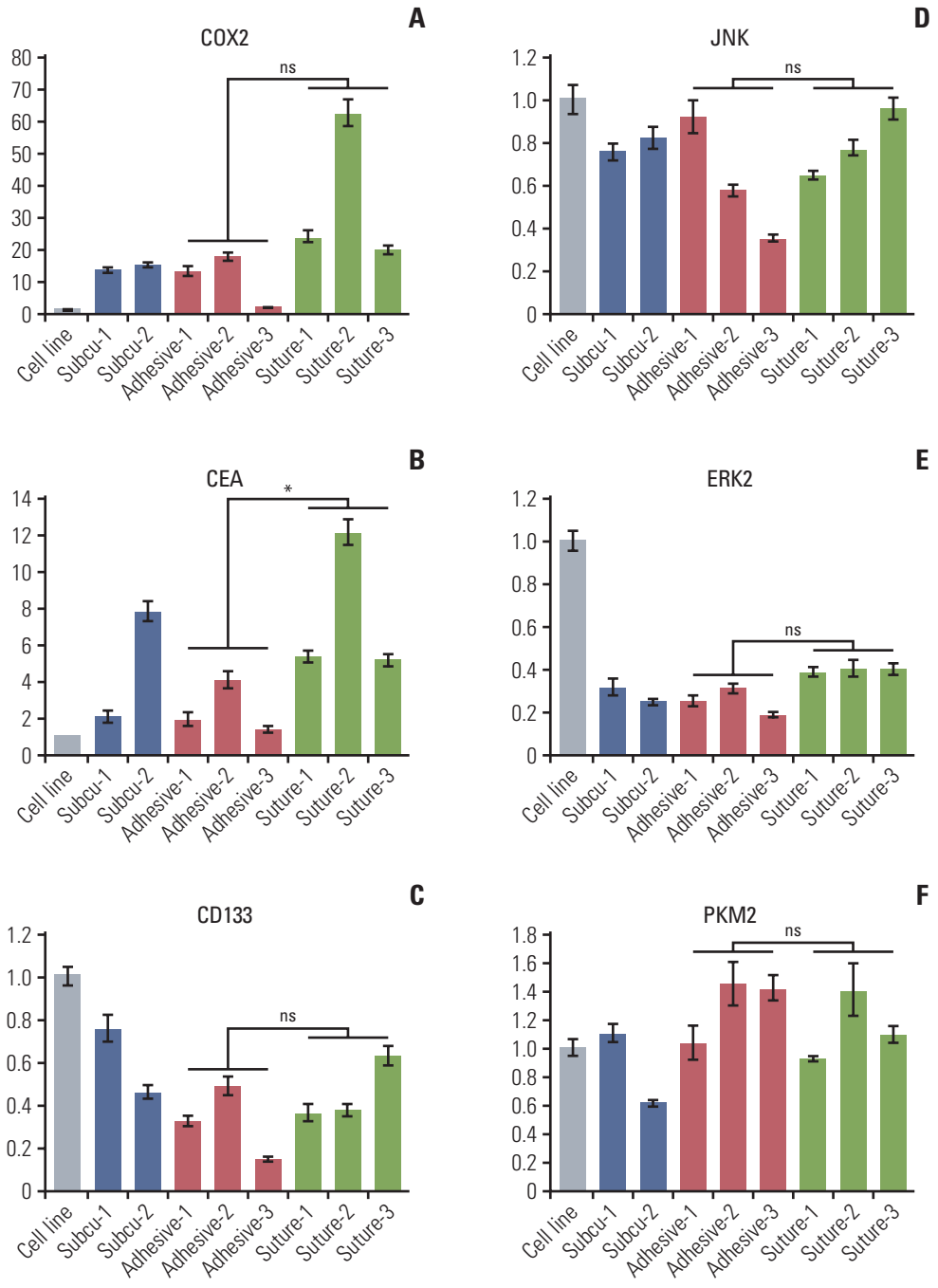

Fig. 4. Real-time PCR analysis from tumors obtained by tissue adhesive method and suture method show similar marker RNA expression pattern. RNA from three tumors of the two graft methods were analyzed by colon tumor markers (A, COX2; B, CEA; C, CD133), proliferation markers (D, JNK; E, ERK2; F, PKM2), cell cycle markers (G, CDK6; H, CCNA2) as well as migration markers (I, IGFBP2). Cell line (HCT-116) and Subcu (tumors from subcutaneous injection) tumor samples were compared together. *$p < 0.05$; n.s., not significant. CCNA2, cyclin A2; CDK6, cyclin-dependent kinase 6; CEA, carcinoembryonic antigen; COX2, cyclooxygenase-2; IGFBP2, insulin-like growth factor binding protein 2. *(Continued to the next page)*

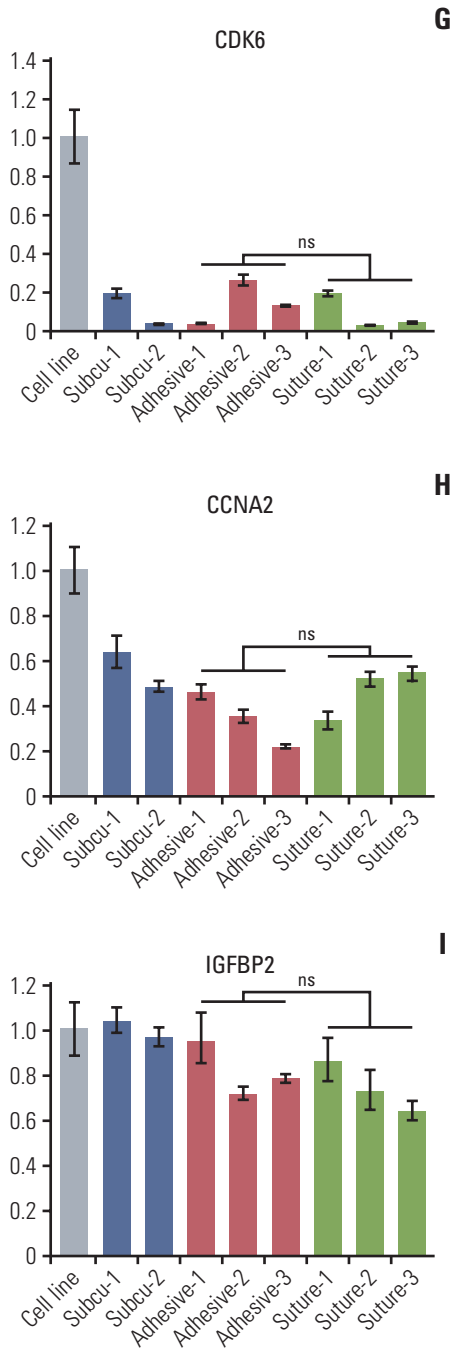

Fig. 4. *(Continued from the previous page)*

ERK RNA were comparable among all groups except for cell lines (Fig. 4E). But the expression of p-MEK was lowest in suture group, whereas p-JNK was lowest in tissue adhesive group (Fig. 5), suggesting suture group has relatively lower MAPK activity and higher apoptotic signal.

Discussion

As reported [4,22-25], the structure of orthotopically implanted tumor tissue plays an important role in the initiation of tumor's attachment, growth, invasion, and distant metastasis. Because of tumor cells are placed in similar microenvironment as the original tumor, the change of intercellular matrix is unlikely to happen. Supporting this idea, orthotopic breast tumor xenografts have been shown to recapitulate the tumor microenvironment and cancer progression better, compared to the subcutaneous xenografts [25]. In colon cancer xenograft mouse model, there was a different response to chemotherapy according to the transplantation site [4]. Thus, the use of orthotopic tumor implantation technique ensures the expression of the clinical features of original tumor.

The present study, demonstrates the tissue adhesive technique can build up the colon cancer model with high success and low motility rate in a shorter time. Based on the lack of microsurgery expertise, suturing a small piece of tumor tissue on the thin mouse colon, by surgical microscope guidance, is still a challenge. Because this technique requires specialized training and suitable instruments [26], many researchers build up the orthotopic cancer model by suturing the tumor tissue onto the cecum of a mouse. One of the reasons for the graft on cecum instead of colon is that the cecum is bigger and has thicker intestinal wall than colon, which will decrease the technical difficulty and perforation rate. In addition, the reported tumor graft rate for orthotopic mouse colon model varies from 70%, which may limit its preclinical utility [22]. In our research, we overcome this limitation by introducing tissue adhesive that does not require suturing. We found there was a significant difference in colon tumor size and procedure duration between the two groups. We guess the reason is that suture line bundling the tissue and colon wall which will compress each other and causes tissue ischemia. There is one mouse with no tumor formation in the suture group, and we speculate this is because the tumor was displaced from the suture site and lost its attachment to colon.

Cyanoacrylate is one of the biodegradable tissue adhesives [27,28]. It is widely used as a method to suture materials for the prevention of infection leakage and seeding of tumor cells in surgery [27]. It binds to the surface within 5-6 seconds in the presence of water or hydroxide ions. Finally, the adhesive will undergo exothermic polymerization within 60 seconds [28]. So, the suitability of cyanoacrylate is another reason for the lower mortality rate in the graft. There are, however, important tips for the use of tissue adhesive *in vivo*. The first is that we should protect the near tissue by gauze covering before giving the adhesive. The second point is that after dropping the tissue adhesive, we should wait 60 sec-

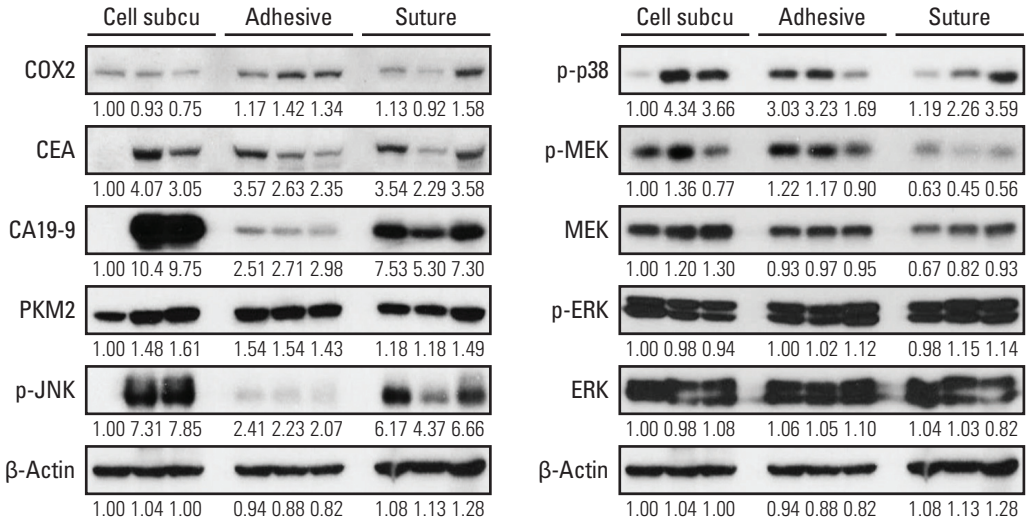

Fig. 5. Western blot analysis of marker expression revealed higher proliferation signal in tissue adhesive with retained tumor marker expression. Protein samples extracted from three tumors of the two graft methods were analyzed by western blot. Cell (HCT-116) and Subcu (tumors from subcutaneous injection) tumor samples were compared together. CA 19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; COX2, cyclooxygenase-2.

onds to make sure the adhesive solidification and then move the tumor into abdomen cavity, that will prevent the adhesive adhere to surrounding tissue.

Our tissue adhesive method makes surgical orthotopic implantation in the colon simpler and shorter time than the conventional suturing techniques. It allows us to finish the procedure of tumor tissue implantation into the ascending colon in less than 5 minutes. The 5 minutes includes opening the abdomen wall, waiting 1 minute to make sure the adhesive solidification and closing the abdomen with a double-layer suture. Because we did not conventionally apply the antibiotic after the procedure, the less mortality in the tissue adhesive group should be attributed to the following factors: (1) shorter time with a lower risk of infection; (2) smaller trauma to the colon with less bleeding; (3) no suture prevents the risk of perforation. So, the tissue adhesive plays a major role in the implantation procedure and it can prevent the bleeding due to colon scratch. Indeed, our pilot experiment to use the tissue adhesive successfully generated pancreatic cancer orthotropic model (S6 Fig.). Based on these data, we think the tissue adhesive method can be an alternative and quick method for tumor implantation in different parts organs, instead of the suturing method.

Previous studies show that although CA19-9 is a biomarker in pancreatic cancer, it can also be recognized as biomarker in colon cancer when combined with CEA [12]. But our study showed that the expression of CA19-9 was quite different with other colon cancer biomarkers, like COX2 and CEA (Fig. 5). The reason of this difference is not clear, but we think CA19-9 is not suitable marker in our model. Instead, using CEA and COX2 together should be a better choice. We also noticed contrast to cell line, CDK6 expression was sharply reduced in all mouse model (Fig. 4G). Given that cyclin D/cdk4(6) complex play a vital role in cell cycle G1 to S transition [29], the decreased regulation of CDK6 would slow cellular proliferation *in vivo*. Besides, we also found that both ERK and p-ERK protein was prominently expressed in all groups, indicating strong activity of MAPK signaling in colon cancer both *in vitro* and *in vivo*. Recent studies showed that high activity of MAPK signaling is induced by increasing growth factors, which consistently restricts tumor cells at the leading tumor edge and displays a progenitor cell phenotype in colon cancer [14]. Therefore, targeting ERK may be a promising way for colon cancer treatment that needed further investigation. Interestingly, we found that there was a remarkable difference of p-JNK among four groups on protein level, with low expression in tissue adhesive group, moderate in suture group and high in subcutaneous group (Fig. 5). Some researches demonstrate that JNK can assist genomic stability and impede tumor formation [30], so this may explain why tissue adhesive group tumor had bigger size and lower rate of metastasis.

Lastly, there are some limitations found in our study. First, small sample size used in this study that might result in bias in interpretation. However, we recently applied this technique to explore the role of nano-stent for colon cancer intervention and found the glue technique consistently and successfully generate orthotropic colon model (manuscript

in preparation). Secondly, after the tumor implantation, no image examination were performed to monitor the tumor. Further study with better cell and detection system will advance our results presented here.

Altogether, we suggest here a tissue adhesive graft as a quick and safe method for establishing a colon cancer orthotopic mouse model. It provides a good alternative way for researchers who are unfamiliar with microsurgical techniques.

This is the first study that described in details using the tissue adhesive method building up the colon tumor tissue orthotopically model. By this method, we improved the graft and survival rate from 60% to 90% quickly and safely compared to suturing method. In addition, the median time required for the procedure was shortened from 11 to 4.5 minutes per mouse. Importantly, we also observed tumor growth is increased significantly in tissue adhesive group. Molecular analysis of tumors from the two methods revealed comparable IHC pattern and the expression of colon tumor markers, as well as proliferation markers. Taken altogether, we propose the tissue adhesive method is a fast and safe way to generate colon orthotopic mouse model.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Ethical Statement

The animal study was approved by the Institutional Animal Care and Use Committee of our institution and adhered to the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Author Contributions

Conceived and designed the analysis: Hu HT, Song HY, Chang S.
Collected the data: Hu HT, Wang Z, Kim MJ.
Contributed data or analysis tools: Hu HT, Wang Z, Kim MJ, Jiang LS, Xu SJ, Jung J, Lee E, Yoon SH, Kim KY.
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Conlficts of Interest

Conflict of interest relevant to this article was not reported.

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