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Title

A33 shows similar sensitivity to but is more specific than CDX2 as an immunomarker of

colorectal carcinoma

Running title

A33 is a more specific CRC marker than CDX2

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Conflict of interest declaration

None to declare

Abstract

Aims

CDX2 is widely used as a sensitive and specific immunomarker for colorectal carcinoma

(CRC) but neither this sensitivity nor specificity is absolute. This study is the first known

comparison of CDX1 and A33 against CDX2 as immunomarkers for CRC.

Methods and Results

As a pilot study, whole sections of 51 cases of liver metastatic carcinoma of different origins

- colorectum (n=32), breast (n=3), oesophagogastric tract (n=4), lung (n=3), pancreas (n=8),

and prostate (n=1) - were immunostained with CDX1, CDX2 and A33. Compared with

CDX1, A33 showed higher sensitivity as a CRC immunomarker, greater interobserver

reproducibility for assessment of expression, and less background cross-reactivity. Therefore,

only A33 was compared with CDX2 for a tissue microarray-based study of primary

adenocarcinomas of different origin: CRC (n=55), liver deposits of metastatic CRC (n=60),

breast (n=101), lung (n=40), oesophagogastric tract (n=134), ovary (n= 67), pancreas (n= 77),

and prostate (n= 56). Combining the whole section and TMA cases of CRC, A33 had a

sensitivity of 95.9% and CDX2 a sensitivity of 97.2%. Combining all the whole section and

TMA cases of non-colorectal carcinomas, A33 showed 85.4% specificity as a marker of CRC

compared to CDX2 which showed a specificity of 64.3%. The higher specificity of A33 as a

colorectal carcinoma immunomarker compared with CDX2 was particularly seen amongst

pancreatic and ovarian carcinomas. Further, unlike with CDX2, none of the prostatic and lung

carcinomas studied showed A33 positivity.

Conclusions

A33 shows similar sensitivity to but is more specific than CDX2 as an immunomarker of CRC.

Keywords

A33, CDX2, immunohistochemistry, colorectal carcinoma, specificity, sensitivity.

Introduction

Carcinoma of unknown primary (CUP) is a common clinical and diagnostic problem.[1, 2] A common presentation of CUP is as liver metastases.[1, 2] Determining the primary neoplasm site giving rise to these metastases has important oncological management implications, especially in the era of personalised medicine. For example, if the carcinoma is of colorectal origin and does not harbour RAS mutations, anti-EGFR therapy becomes a potential management option.[3] Outside radiological and endoscopic investigations, the main way in which cases of CUP are further investigated is tissue based.[1, 2] This involves morphological assessment of a biopsy of the metastasis and in particular, immunohistochemical analysis of the carcinoma.[1] It is standard practice to apply a panel of antibodies to CUP tissue to help determine its likely site of origin.[1] As a specific and sensitive immunomarker for colorectal carcinoma (CRC), CDX2 is one of the most commonly used stains in the investigation of CUP.[1] Nonetheless, its sensitivity has been recorded to range from 100% to 71%;[4-7] poorly differentiated CRC, in particular, may lack CDX2 expression.[4, 5] Further, it is now well recognised that CDX2 expression may been seen in a varying proportion of pancreaticobiliary, ovarian and particularly oesophagogastric carcinomas and even, rarely, amongst lung and prostatic carcinomas.[4-7]

One of the study authors previously investigated the roles of CDX2 and CDX1 in the pathogenesis of Barrett's metaplasia and also the potential for A33 to serve as a marker of such metaplasia.[8, 9] The similarity of CDX1 to CDX2 as a homeobox protein regulating intestinal differentiation[10, 11] suggests the former may also serve as an immunomarker for CRC. Bai and colleagues raised their own monoclonal antibody to CDX1 but only tested it amongst gastric carcinomas.[12] Similarly, only gastric carcinomas were studied in a later study which used a polyclonal CDX1 antibody.[13] The fact that CDX1 may regulate A33 (also known as gpA33) expression[11] and that physiological expression of A33 is almost limited to intestinal epithelium[14, 15] suggests that A33 might also serve as an immunomarker for CRC. Indeed, haemagglutination testing of an early A33 antibody (clone mAbA33) amongst cancer cell lines showed reactivity in several CRC cell lines, one of three pancreatic carcinoma cell lines and one of 24 lung carcinoma cell lines but not in any ovarian or breast carcinoma cell lines.[16] Sakamoto and colleagues also studied A33 expression amongst gastric carcinomas but no other carcinoma sites.[16] Using the same antibody, an earlier study published in 1996 recorded A33 expression amongst 95% of CRC and at least 50% of gastric and pancreatic carcinomas but in less than 10% of breast and ovarian carcinomas.[14] We are unaware of any subsequently published data on A33 immunopositivity amongst a range of adenocarcinomas from different organs.

Our previous intentions to study CDX1 and A33 as immunomarkers for CRC were hampered by the limited availability of monoclonal antibodies to the proteins which could be applied successfully to immunohistochemistry. More recently, however, the previous laboratory of one of the study authors has successfully raised a monoclonal antibody to CDX1[17] and there are now commercial A33 monoclonal antibodies marketed for immunohistochemical use. The following study therefore, represents the first known attempt to systematically compare CDX1 and A33 with CDX2 as immunomarkers of CRC.

Materials and Methods

Two cohorts of different samples were used. The initial pilot study used anonymised tissue samples that had been complied and analysed in a previous morphology-based study.[18] All of these cases were liver metastases from known or subsequently established sites of origin.[18] These cases had all been collected from the files of the Department of Histopathology at the Bristol Royal Infirmary and all yielded whole tissue sections for immunohistochemistry. The second cohort of tissue samples were all tissue microarray (TMA) sections. These TMA sections were derived from either the NHS Greater Glasgow and Clyde Bio-Repository in Glasgow or the Department of Pathology at the Royal Infirmary of Edinburgh. Each TMA represented primary adenocarcinoma from one particular anatomical site, i.e. breast, lung, ovary, oesophagogastric tract, pancreas, prostate or colorectum. Further, the latter had a paired TMA of matched liver metastases. This study and its use of all these anonymised tissue samples had received research ethics committee approval (REC Oxford - South Central; reference number: 14/SC/0155; 11 Dec 2015).

All immunohistochemistry was performed with the Leica Bond III automated immunostainer (Leica Biosystems, Newcastle-upon-Tyne, UK) using the Bond polymer refine kit detection system (Leica Biosystems). The CDX1 monoclonal antibody (obtained from the Cancer and Immunogenetics Laboratory, Oxford, UK) was used at a 1/200 dilution (incubation period of 12 minutes) after heat pretreatment with Bond epitope retrieval solution 2 (Leica) for 20 minutes. The CDX2 monoclonal antibody (clone EP25, Leica Biosystems) was used commercially prediluted (incubation period of 15 minutes) after heat pretreatment with Bond epitope retrieval solution 2 (Leica Biosystems) for a duration of 20 minutes. The A33 monoclonal antibody (clone EPR4240, Epitomics/Abcam, Cambridge, UK) was used at a

1/250 dilution (incubation period of 15 minutes) after heat pretreatment with pH6 citrate buffer for 20 minutes. A positive control (of normal large bowel wall including mucosa) was run with each round of each antibody.

Both the whole tissue sections and the TMA sections were scored for intensity and proportion of immunopositivity. Intensity was scored as absent, strong (equivalent intensity to the positive control tissue) or weak (any intensity below that of the positive control tissue). For the whole tissue sections, a proportion of positivity was scored using a five point scale: 0 (0%); 1 (1-25%); 2 (26-50%); 3 (51-75%); and 4 (76-100%). For the TMA sections, a proportion of positivity was scored using a three point scale: 0 (0%); 1 (1-50%); and 2 (51-100%). The immunostained whole sections were assessed independently by two observers (NW and JC for CDX1 and CDX2; NW and SE for A33), and the immunostained TMA sections were assessed by one observer (NW).

Results

Amongst normal large bowel wall and all the tested tissues (both whole sections and TMA sections), A33 showed only membranous positivity. In the normal large bowel wall, this positivity was restricted to crypt and surface epithelium (diffusely throughout the entire length of the crypt) and was not seen amongst any other cell type. There was no A33 positivity amongst submucosal or deeper tissues.

 CDX1 positivity amongst non-neoplastic intestinal epithelium and amongst most carcinomas (see below) was of nuclear location (Figure 1). Rarely (see below), CDX1 positivity amongst carcinomas had a membranous location instead (Figure 1). In the positive control and the test cases, CDX1 immunohistochemistry often showed cytoplasmic positivity amongst smooth muscle (muscularis mucosae, muscularis propria and vascular media) and amongst myofibroblasts (Figure 1).

Fifty-one cases of liver metastatic carcinoma (see the Table for primary sites of origin) were used for the pilot study involving immunohistochemistry for CDX1, CDX2 and A33. Amongst these 51 whole tissue sections, there was 100% concordance between two independent assessors when recording the presence (defined as strong or weak intensity positivity) versus absence of immunopositivity for A33. Similar assessment of the CDX1 immunostained sections yielded two cases with discordant findings (i.e. presence versus absence of positivity) between two assessors, and similar assessment of the CDX2 stained sections yielded one case with discordant findings between two assessors.

The Table presents the sensitivities and specificities of A33, CDX2 and CDX1 as immunomarkers of colorectal carcinoma, derived from this pilot study of 51 cases. The non-colorectal carcinomas which most commonly showed positivity for all three antibodies, derived from the pancreas or the oesophagogastric tract (Table). While only one pancreatic carcinoma showed CDX1 nuclear positivity, four other pancreatic carcinomas showed membranous CDX1 positivity (Figure 1). Such membranous location of CDX1 positivity was not seen amongst any of the other carcinomas in this pilot study cohort.

CDX1 was abandoned and only A33 compared with CDX2 for the TMA-based analysis for several reasons related to this pilot study data. First, the inter-observer reproducibility for A33 assessment was higher than that for CDX1. Second, this reproducibility difference may have related to the much cleaner immunohistochemical staining of A33 (compared with CDX1) and/or the apparent cross-reactivity of the CDX1 antibody with smooth muscle. Third, A33 showed equivalent sensitivity as a CRC immunomarker to CDX2 and such sensitivity was higher than for CDX1. Fourth, the fact that A33 positivity was of membranous location (compared to the nuclear location of CDX1 positivity) opened the

possibility for combined immunostaining with both A33 and CDX2, e.g. using dual labelling. As a final note, it is worth acknowledging data showing that CDX1 regulates A33 expression[11] and therefore, A33 can be serving as a surrogate for CDX1 expression anyway.

A33 and CDX2 immunohistochemistry was performed on sections cut from eight TMA blocks (Table). Two blocks represented primary colorectal carcinoma and liver deposits of metastatic colorectal carcinoma. Between these two TMA blocks, there were a total of 39 matched primary and secondary colorectal carcinoma tissues.

Combining the liver metastatic colorectal carcinoma cases (whole section and TMA sections, Table), both A33 and CDX2 show identical sensitivity as a marker of colorectal carcinoma, i.e. 96.7% (Figure 2). The same two cases of liver metastatic colorectal carcinoma were A33 and CDX2 negative. If the primary colorectal carcinoma cases were combined with the liver metastatic cases (total of 147 specimens, including 39 matched primary and secondary specimens), A33 had a sensitivity of 95.9% and CDX2 a sensitivity of 97.2%.

Out of 30 whole sections of metastatic colorectal carcinoma which showed A33 positivity and CDX2 positivity, only one case showed weak intensity A33 positivity and only one case showed weak intensity CDX2 positivity, but four cases showed <50% A33 positivity whereas only one case showed <50% CDX2 positivity. Of the 59 TMA cases of metastatic colorectal carcinoma showing A33 positivity and CDX2 positivity, two and no cases showed weak intensity positivity for A33 and CDX2 respectively, whereas 14 and 3 cases showed <50% positivity for A33 and CDX2 respectively. Amongst the 39 cases of matched primary and metastatic colorectal carcinoma in the TMA cases, all 39 pairs showed CDX2 positivity in

both primary and metastatic tissue, and all but one case showed A33 positivity in both primary and metastatic tissue. The discordant A33 case showed positivity in the primary neoplasm but not in its matched metastatic tissue.

Combining non-colorectal adenocarcinoma cases from the whole section specimens and the TMA specimens (Table), A33 showed 85.4% specificity as a marker of colorectal carcinoma compared to CDX2 which showed a specificity of 64.3%. If oesophagogastric carcinomas were re-categorised for analysis, A33 showed 97.8% specificity as a marker of carcinoma arising from the tubular gastrointestinal tract compared with CDX2 which had a specificity of 79.5%. The differences in specificity were mainly due to the rarity of A33 positivity amongst pancreatic and ovarian carcinomas compared with CDX2 (Figure 2). However and in addition, CDX2 positivity was occasionally seen amongst prostatic and lung carcinomas, whereas all these carcinomas studied were negative for A33 (Figure 2).

Discussion

This study has demonstrated that A33 is a more specific immunomarker for colorectal carcinoma than CDX2.

We considered whether this higher specificity was just due to a lower sensitivity. Amongst the primary colorectal carcinoma tissue in the TMA block, there were two cases which were CDX2 positive but were A33 negative. Further, the proportions of colorectal carcinoma cells showing A33 positivity were potentially lower than those showing CDX2 positivity. This potential difference in proportions was more apparent in the TMA cores and less striking in whole tissue sections, but it is acknowledged that diagnostic immunomarkers for CUP are commonly applied to biopsies and aspirates[1] rather than large resection derived whole tissue

sections. Finally, compared with CDX2 which showed complete concordance of staining amongst 39 pairs of matched primary and metastatic colorectal carcinoma, one case showed A33 positivity amongst the primary carcinoma but not amongst the matched metastatic tissue. Nonetheless and despite all the above, both in the whole section cases and in the TMA cases of liver metastatic colorectal carcinoma, A33 still showed an overall identical sensitivity to CDX2. In conclusion, while the sensitivity of A33 might be, at most, marginally less than that of CDX2, this was not demonstrated amongst metastatic CRC, and we regard this potential difference as too small to completely explain the higher specificity shown by A33.

We also considered whether the higher specificity of A33 might be due to erroneously higher CDX2 positive rates being recorded amongst our non-colorectal carcinomas. However, our proportions of CDX2 positive non-CRC cases were comparable to previously published data: 75% of our oesophagogastric adenocarcinomas compared with 70%[7] and 70%;[6] 38% of our pancreatic adenocarcinomas compared with 32%[7] and 50%;[6] 9% of our prostatic adenocarcinomas compared with 4%[7] and 2%;[6] and 7% of our lung adenocarcinomas compared with 0%[7] and 6%.[6] Werling and colleagues recorded CDX2 positivity in 9/14 (64%) mucinous ovarian carcinomas and 0/36 non-mucinous ovarian carcinomas,[7] whereas Moskaluk and colleagues recorded CDX2 positivity in 1/4 (25%) mucinous ovarian carcinomas and 6/55 (11%) non-mucinous ovarian carcinomas.[6] It is more difficult to compare our 49% CDX2 positivity rate amongst ovarian carcinomas with these published data because our TMA cases were not further subtyped. However, the fact that a large proportion of our TMA ovarian cases were CDX2 positive suggests that many were likely to be mucinous carcinomas.

As an immunomarker of colorectal carcinoma tested in the pilot study, CDX1 showed similar specificity to A33, but less sensitivity compared to A33 and CDX2. Even if the tested CDX1 antibody was to be further assessed for diagnostic use, its apparent cross-reactivity with smooth muscle would still have to be addressed. Indeed, the overall much cleaner staining of the A33 antibody tested would currently favour it for diagnostic use over the CDX1 antibody. The membranous CDX1 positivity shown by a proportion of our pancreatic carcinomas is a novel finding. However, applying this to diagnostic practice may be limited by potential confusion between this membranous staining and the cross-reacting cytoplasmic staining shown by the CDX1 antibody.

The superiority of A33 as a specific immunomarker for colorectal carcinoma compared with CDX2 was seen primarily amongst pancreatic and ovarian carcinomas. We did often find CDX2 positivity to be weaker and/or focal in pancreatic and ovarian cancers compared with CRC (data not shown). However, in diagnostic practice and in our experience, it is challenging to use these more subjective assessments of proportion and/or intensity of immunopositivity to confidently subtype CUP. Our A33 expression profile amongst adenocarcinomas of differing origin is similar to that recorded by Garincesha and colleagues apart from their pancreatic carcinoma data.[14] It is difficult to be certain why A33 expression was found amongst 50% of their pancreatic carcinomas[14] compared with only 4% of our pancreatic carcinomas. The earlier study used a mouse monoclonal antibody on presumed whole tissue sections from frozen samples[14] whereas we used a rabbit monoclonal antibody on a tissue microarray prepared from formalin fixed paraffin embedded blocks. However, these differences are unlikely to explain a differential staining pattern just for pancreatic carcinomas and not for carcinomas of other organ sites. The exact A33 epitopes used to raise the two antibodies are not known or have not been published, therefore precluding a direct

comparison of the two antibodies. However, it may be of relevance that the earlier mAbA33 antibody was raised through inoculation of a BALB/c mouse with the ASPC-1 pancreas cancer cell line and SP2/0 myeloma cells.[14]

The few reported studies of A33 expression amongst human and murine physiological tissues have never mentioned any expression in the normal pancreas.[14, 15] One of these studies reported that "areas of reactive normal pancreatic ducts adjacent to the pancreatic cancers also showed scattered A33+ epithelial cells" but it may be speculated that these areas were, in fact, foci of pancreatic intraepithelial neoplasia (PanIN). By contrast, multiple studies have described CDX2 expression by at least a subset of pancreatic ductal cells.[5-7] This difference in physiological expressions may, at least in part, explain why A33 is less often expressed by pancreatic carcinomas than CDX2. Pancreatic adenocarcinomas (referred to usually as ductal carcinomas) are thought to arise from PanIN, intraductal papillary mucinous neoplasm or more rarely mucinous cystic neoplasm.[19] Therefore, it would be interesting to see what proportions of these precursor lesions express CDX2 versus A33.

The fact that both A33 and/or CDX2 are expressed by a large proportion of oesophagogastric adenocarcinomas is not surprising as many of these tumours arise on a background of intestinal metaplasia (in atrophic gastritis) or Barrett's metaplasia.[20, 21] Our proportion of A33 positive oesophagogastric adenocarcinomas (46%) was similar to the proportions of gastric carcinomas previously described to express A33, i.e. 45%[16] and 58%.[14] The fact that a proportion of oesophagogastric carcinomas may arise instead from non-intestinal precursor lesions (e.g. foveolar-type dysplasia)[22] might explain why a proportion of these neoplasms do not express A33 and/or CDX2. However, it is still difficult to explain why a lower proportion of these carcinomas express A33 compared with CDX2. CDX2 is not known to be expressed

by physiological gastric mucosa[5, 6] and, if anything, it is more controversial whether or not A33 is expressed in such mucosa. In mice, A33 has been demonstrated along the entire gastric gland though only in the pylorus.[15] One of this study's authors previously demonstrated A33 mRNA in one of four body gastric biopsies[9] but it is conceivable that that particular biopsy included intestinal metaplasia. Further, Sakamoto and colleagues reported no A33 immunostaining in 38 cases of "foveolar epithelium and gastric glands".[16]

Further lines of research are required to clarify the preliminary data of this study and also to explore more specific questions raised by these data. It is the mucinous variant of ovarian carcinoma which is most difficult to distinguish from gastrointestinal carcinoma including colorectal carcinoma.[1] While many of our TMA ovarian carcinomas were likely to be mucinous (for reasons explained above), a more targeted analysis of only primary ovarian mucinous carcinoma with A33 is required. Another diagnostic scenario which can be periodically challenging is the distinction between colorectal carcinoma and primary bladder adenocarcinoma, particularly as the latter can show CDX2 expression.[7] It has been suggested that beta catenin immunohistochemistry (nuclear decoration seen only in CRC[23]) may help make this distinction, but whether A33 may also aid in this distinction should be investigated next. Finally, larger cohorts of colorectal carcinoma from other centres should also be studied with A33, particularly to confirm the immunomarker's sensitivity and especially in biopsy material. One particular cohort worth studying are CDX2 negative CRCs which may represent a specific subgroup with poorer prognosis.[24] Accepting these are only limited data, the three CDX2 negative CRCs in our study cohort all also lacked A33 expression.

While the above further research is required, this study's data show great promise for A33 as both a sensitive and more specific marker of colorectal carcinoma than CDX2. Therefore, A33 may serve at least as an adjunct to CDX2 in the immunohistochemical panel used routinely to investigate CUP.

Acknowledgements

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Table

Primary site of carcinoma	Whole tissue section cases			Tissue microarray cases	
	A33	CDX2	CDX1	A33	CDX2
Large bowel (primary)	n/a	n/a	n/a	52/55* (95%)	54/55* (98%)
Large bowel (metastatic)	30/32 (94%)	30/32 (94%)	29/32 (91%)	59/60* (98%)	59/60* (98%)
Breast	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/104 (0%)	0/104 (0%)
Lung	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/43 (0%)	3/43 (7%)
Oesophagogastric	2/4 (50%)	3/4 (75%)	1/4 (25%)	64/138 (46%)	103/138 (75%)
Ovary	n/a	n/a	n/a	5/67 (7%)	33/67 (49%)
Pancreatic	1/8 (13%)	1/8 (13%)	1/8 (13%)	3/85 (4%)	32/85 (38%)
Prostate	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/57 (0%)	5/57 (9%)
Specificity as a colorectal carcinoma marker	84%	79%	89%	85%	64%

* 39 of the tissue microarray colorectal carcinoma cases had matched primary and metastatic tissue.

Table and Figure legends

Table

Immunostaining patterns of CDX2, CDX1 and A33 amongst the whole tissue section cases and/or tissue microarray cases.

Figure 1

CDX1 immunohistochemistry of A) colorectal carcinoma and B) pancreatic ductal carcinoma. While the colorectal carcinoma shows only nuclear positivity, decoration of vascular media (arrow head) and myofibroblasts (arrows) of the desmoplastic stroma was of a cytoplasmic location. The pancreatic carcinoma was one of four (out of 8 pancreatic carcinomas studied) that showed striking membranous CDX1 positivity; this cellular location of positivity was not seen amongst carcinomas from any other site of origin.

Figure 2

A33 and CDX2 immunohistochemistry performed on paired microarray tissue of: A & B) a colorectal adenocarcinoma; C & D) an ovarian carcinoma; E & F) a lung adenocarcinoma; and G & H) a pancreatic adenocarcinoma. The colorectal carcinoma shows diffuse positivity for both A33 and CDX2 (combined nuclear and cytoplasmic CDX2 positivity amongst colorectal carcinoma is a recognised phenomenon[5]). The A33 staining of the colorectal carcinoma is only membranous in location. The ovarian carcinoma, lung adenocarcinoma and pancreatic adenocarcinoma each show CDX2 positivity but no evidence of A33 positivity.

| A33 | CDX2 |

Colorectum

A

B

Ovary

C

D

Lung

E

F

Pancreas

G

H