

## REVIEW

## The links between axin and carcinogenesis

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The products of the two mammalian Axin genes (Axin1 and its homologue Axin2) are essential for the degradation of  $\beta$  catenin, a component of Wnt signalling that is frequently dysregulated in cancer cells. Axin is a multidomain scaffold protein that has many functions in biological signalling pathways. Overexpression of axin results in axis duplication in mouse embryos. Wnt signalling activity determines dorsal–ventral axis formation in vertebrates, implicating axin as a negative regulator of this signalling pathway. In addition, Wnts modulate pattern formation and the morphogenesis of most organs by influencing and controlling cell proliferation, motility, and fate. Defects in different components of the Wnt signalling pathway promote tumorigenesis and tumour progression. Recent biochemical studies of axins indicate that these molecules are the primary limiting components of this pathway. This review explores the intriguing connections between defects in axin function and human diseases.

Axin was originally identified as the product of the mouse gene called “fused” or fu (renamed Axin), and has since been shown to play a crucial role in controlling axis formation during embryonic development. Axin overexpression in frog embryos inhibits dorsal axis formation. Furthermore, mutation of the mouse gene “fused” was found to cause axis duplication in homozygous mouse embryos.<sup>1</sup> Wnt signalling activity determines dorsal–ventral duplication in vertebrates and these results suggested that Axin somehow negatively regulates this signalling pathway. The subsequent demonstration of the effect of Axin on  $\beta$  catenin concentrations, together with its biallelic inactivation in some human hepatocellular carcinomas (HCCs), indicated that Axin is a tumour suppressor gene.<sup>2</sup> To date, axin has been implicated in at least three different signalling pathways: the stress activated protein kinase (SAPK), transforming growth factor  $\beta$  (TGF $\beta$ ), and Wnt signalling pathways (fig 1). The multimeric nature of axin complexes suggests that axin might play an important role in other cell signalling systems, in addition to the coordination of these signals.

“The effect of Axin on  $\beta$  catenin concentrations, together with its biallelic inactivation in some human hepatocellular carcinomas, indicated that Axin is a tumour suppressor gene”

## WNT SIGNALLING

The Wnt signalling pathway regulates cellular proliferation, differentiation, and motility and is essential for development and morphogenesis.<sup>3–5</sup> Alterations in protein phosphorylation status are central to the regulation of Wnt signalling. Several components of the Wnt signalling pathway—including axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3; both GSK $\beta$  and GSK $\alpha$ ), and  $\beta$  catenin—are phosphoproteins that are regulated through phosphorylation. In unstimulated cells, GSK3 $\alpha$  and GSK3 $\beta$  phosphorylate cytoplasmic  $\beta$  catenin, which creates a recognition motif for  $\beta$  transducin repeat containing protein, an E3 ubiquitin ligase.<sup>6</sup> The ubiquitinated  $\beta$  catenin is rapidly degraded by the 26S proteasome, ensuring that cytoplasmic concentrations of  $\beta$  catenin are very low. Secreted Wnt ligands act on the cell surface receptor Frizzled and activate Dishevelled (DVL) through a poorly understood mechanism. Activated DVL binds and inhibits the phosphorylation of  $\beta$  catenin by GSK3 $\beta/\alpha$ , blocking  $\beta$  catenin degradation (fig 2), so that  $\beta$  catenin accumulates and translocates to the nucleus, where it interacts with the T cell specific factor (TCF)/lymphoid enhancer binding factor 1 (LEF-1) transcription factor and induces the transcription of target genes such as c-jun, c-myc, and cyclin D1.<sup>7–11</sup> Several in vitro and in vivo studies suggest that axins serve as scaffold proteins that bind directly to many proteins involved in the Wnt signalling pathway, and promote the phosphorylation of  $\beta$  catenin by driving the formation of a complex with APC and GSK3.<sup>1 12–14</sup>

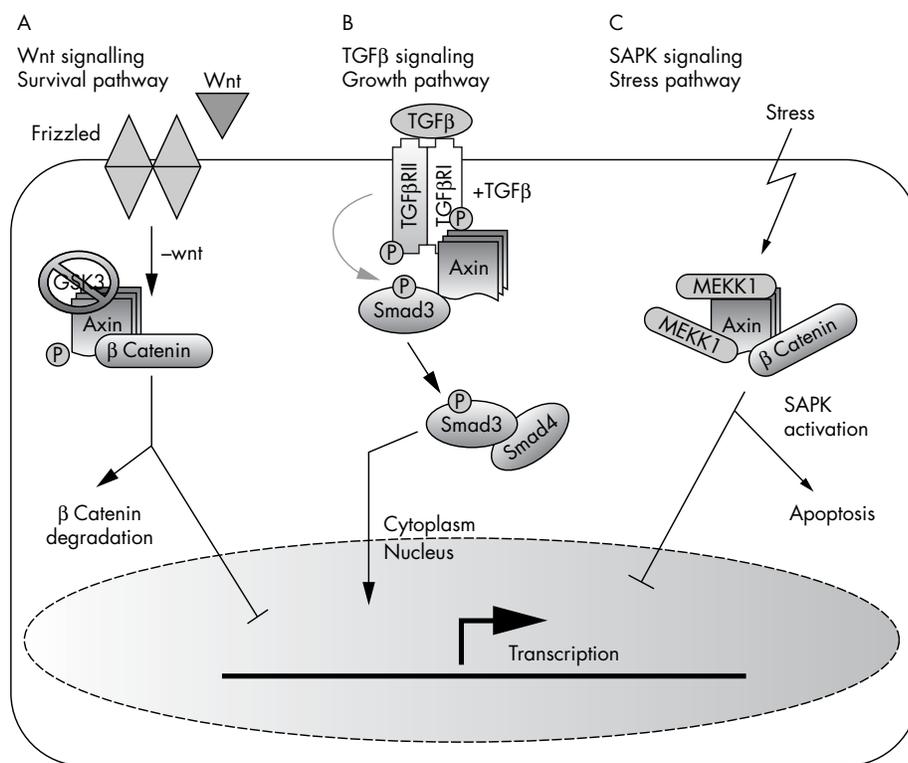
The  $\beta$  catenin molecule is crucial in this pathway and shows abnormal expression and localisation in a wide variety of human cancers.<sup>15</sup> In recent years, so called “canonical” Wnt signalling has received considerable attention because many components of this signalling cascade have been shown to play a role in tumorigenesis.<sup>1</sup> The concentration of  $\beta$  catenin in cells is tightly regulated by the APC–axin–GSK3 destruction complex.<sup>16</sup> APC, another component

**Abbreviations:** aa, amino acids; AD, Alzheimer’s disease; APC, adenomatous polyposis coli; CKI/II, casein kinase I/II; DIX, Dishevelled and axin binding domain; DVL, Dishevelled; Frat, frequently rearranged in activated T cells; GSK3, glycogen synthase kinase 3; HB, hepatoblastoma; HCC, hepatocellular cancer; JNK, Jun N-terminal kinase; LEF-1, lymphoid enhancer binding factor 1; MB, medulloblastoma; MEKK1, mitogen activated protein/extracellular regulated kinase kinase kinase 1; MID, MEKK binding domain; PP2A, protein phosphatase 2A; RGS, regulator of G protein signalling; SAPK, stress activated protein kinase; SCC, oesophageal squamous cell carcinoma; TCF, T cell specific factor; TGF $\beta$ R, transforming growth factor  $\beta$  receptor

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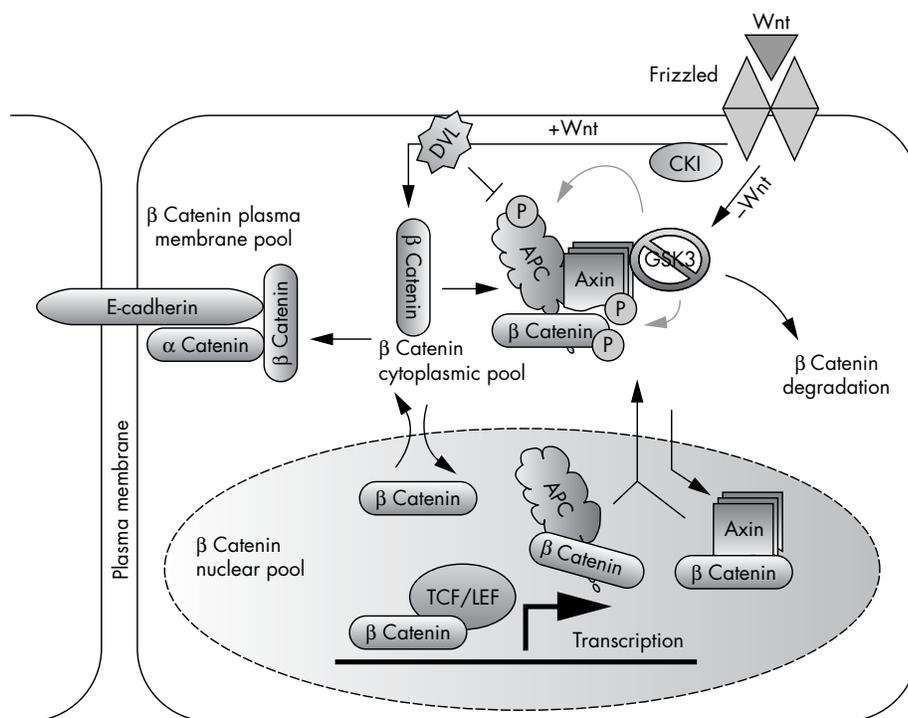
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**Figure 1** At least three different signalling pathways are regulated by axin. (A) In the absence of Wnt ligands, axin stimulates  $\beta$  catenin phosphorylation and subsequent protease mediated degradation limits its transcriptional activity. (B) In the presence of transforming growth factor  $\beta$  (TGF $\beta$ ) signals, axin stimulates Smad phosphorylation by TGF $\beta$  receptors (TGF $\beta$  receptors I and II). The activated Smads then translocate to the nucleus and activate transcription of downstream target genes. (C) In cells subjected to stress, axin binds to mitogen activated protein/extracellular regulated kinase kinase kinase 1 (MEKK1) and stimulates stress activated protein kinase (SAPK)/JNK (Jun N-terminal kinase) mediated apoptosis. GSK3, glycogen synthase kinase 3.

of Wnt signalling, is mutated in most (~ 70%) human colorectal cancers and is also linked to familial adenomatous polyposis.<sup>17</sup> Inappropriate activation of Wnt signalling by mutation of different components of this pathway has been seen in a large number of other human cancers including colon carcinoma,<sup>18-19</sup> medulloblastoma,<sup>20-21</sup> melanoma,<sup>22</sup> hepatocellular carcinoma,<sup>23</sup> and ovarian and uterine cancer.<sup>24-26</sup> Although both  $\beta$  catenin and Wnt are positive regulators of

the pathway and have been identified as protooncogenes, APC and axin are considered as negative effectors of the pathway and function as tumour suppressors.<sup>27</sup> This model asserts that because axin-APC-GSK3 mediated degradation of  $\beta$  catenin is inhibited by either Wnt signals or mutation of components of the Wnt signalling pathway, the concentration of  $\beta$  catenin in cells is raised, leading to increased cell proliferation and cancer.



**Figure 2** The role of axin in Wnt signalling. The Wnt signalling pathway plays an important role in the regulation of cellular proliferation, differentiation, motility, and morphogenesis. Axin serves as a scaffold protein that binds many of the proteins involved in this pathway. In the absence of Wnt ligands, the  $\beta$  catenin destruction complex, which is composed of adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and axin, is formed and leads to the phosphorylation and degradation of  $\beta$  catenin. In the presence of Wnt ligands, the formation of this complex is inhibited and the now stabilised  $\beta$  catenin is translocated into the nucleus and activates the transcription of downstream target genes. Axin is also implicated in shuttling  $\beta$  catenin out of the nucleus. However,  $\beta$  catenin can move in and out of the nucleus independently of axin. It is unclear whether axin participates in the regulation of all of the different pools of  $\beta$  catenin in the cell. Deregulation of many components of the Wnt pathway has been found in human cancer. DVL, Dishevelled; LEF, lymphoid enhancer binding factor; P, organic phosphate; TCF, T cell specific factor.

## TGF $\beta$ SIGNALLING

Similar to the Wnt signalling pathway, TGF $\beta$  signalling regulates several cellular functions, often in concert with Wnt signalling, including proliferation, differentiation, migration, and apoptosis.<sup>28</sup> TGF $\beta$  also plays an important role in carcinogenesis. Upon stimulation, TGF $\beta$  receptors phosphorylate Smads—the TGF $\beta$  effector proteins (fig 1B).<sup>29</sup> The phosphorylated Smads then translocate into the nucleus, where they regulate the transcription of target genes. Although TGF $\beta$  can often appear to have different effects in different cell types, its primary effect on colonic epithelial cells is to reduce proliferation and induce differentiation.<sup>30</sup> Mutations of the TGF $\beta$  receptors are often found in cancer cells with defects in mismatch repair systems.<sup>31</sup> Axin has been shown to regulate the TGF $\beta$  signalling pathway by acting as an adaptor for Smad3, one of the TGF $\beta$  effectors (fig 1B).<sup>32</sup> Both axin1 and axin2 physically interact with Smad3 through interaction with a domain that is located between the  $\beta$  catenin and DVL binding domains within the C-terminal region. The binding of DVL to axin has been shown to inhibit axin mediated downregulation of  $\beta$  catenin. Although both Smad3 and DVL interact with the same region of axin, biochemical studies have shown that the interaction of DVL with axin does not compete with Smad3 binding to axin. Colocalisation of axin with Smad2 and Smad3 in the cytoplasm has been observed. Upon receptor activation, Smad3 bound to axin is efficiently phosphorylated by TGF $\beta$  receptor 1 and dissociates from the axin complex. Thus, axin may facilitate the phosphorylation and transcriptional activity of Smad3.<sup>32</sup> TGF $\beta$  is one of the anti-oncogenic factors that inhibit nuclear  $\beta$  catenin signalling in the Wnt pathway. Smad4, another effector of TGF $\beta$ , has been shown to facilitate Wnt signalling through interaction with  $\beta$  catenin and TCF/LEF.<sup>33</sup> Although axin is a negative regulator of the Wnt signalling pathway, it facilitates TGF $\beta$  signalling. These results indicate that axin has a dual function in signal transduction—acting as a negative regulator of the Wnt signalling pathway and as a positive regulator of the TGF $\beta$  signalling pathway. Deregulation of both pathways is frequently detected in human cancer cells.

## SAPK/JNK SIGNALLING

The SAPK/JNK (stress activated protein kinase/Jun N-terminal kinase) signal transduction pathway is activated in cells in response to stress. It is also involved in many normal physiological processes including tissue morphogenesis, cell proliferation, cell survival, and cell death.<sup>34</sup> Depending on the cell type or the context of activation of other signalling pathways, SAPK/JNK can help mediate cell survival or apoptosis.<sup>35–36</sup> Overexpression of axin1 in cells stimulates SAPK/JNK and can induce apoptosis through the activation of MEKK (mitogen activated protein/extracellular regulated kinas kinase).<sup>37–38</sup> A MEKK1 interacting domain (MID) on axin is flanked by the APC and GSK3 binding sites (fig 3A), and the C-terminal region of axin, which contains an oligomerisation domain, is necessary for SAPK activation (fig 3A). The effect of axin on the induction of apoptosis is mediated by SAPK/JNK activation and the destabilisation of  $\beta$  catenin.<sup>39</sup> The importance of the axin–MEKK interaction and subsequent SAPK/JNK activation has been shown by the Axin1 mutations found in cancer cells that are resistant to apoptosis.<sup>2–39–41</sup> Induced expression of Axin in transgenic mice leads to massive cell death in different organs as a consequence of the increased capacity to degrade  $\beta$  catenin.<sup>42</sup> The introduction of wild-type Axin1 into hepatocellular and colorectal cancer cells induces apoptosis in a SAPK/JNK dependent manner. GSK3 competes with a MEKK binding site on axin1 and thereby inhibits SAPK/JNK induced apoptosis. This is consistent with observations that

GSK3 can prevent cell death under certain circumstances.<sup>43</sup> Thus, axin can bind to either GSK3 or MEKK1 and form distinct complexes with opposing effects on apoptosis. Although axin can be “switched” to activate the SAPK/JNK pathway instead of Wnt signalling, it is still capable of downregulating  $\beta$  catenin.<sup>44</sup> Axin domains essential for Wnt signalling activation are distinct from those domains required for SAPK/JNK activation.

“Depending on the cell type or the context of activation of other signalling pathways, SAPK/JNK can help mediate cell survival or apoptosis”

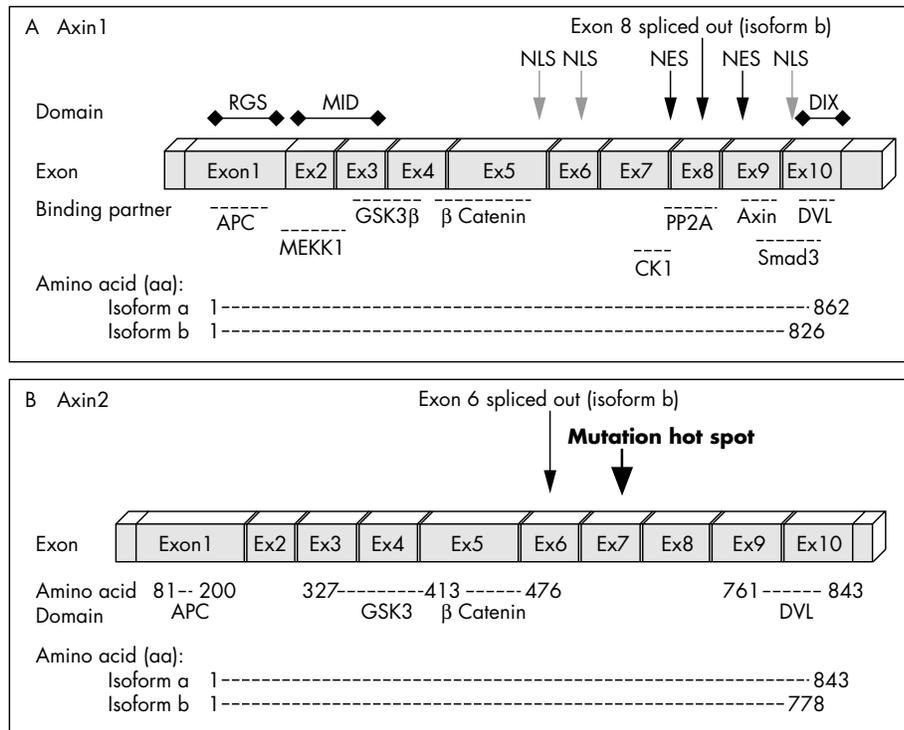
MEKK4, which is related to MEKK1, was recently identified as another axin binding protein that mediates SAPK/JNK activation.<sup>45</sup> Interestingly, MEKK1 and MEKK4 compete for axin binding, even though the binding domain for MEKK4 is separate from the MEKK1 binding domain.<sup>46</sup> Axin dimerisation and oligomerisation appear to be necessary for the activation of the SAPK/JNK pathway.<sup>37</sup> Other components of Wnt signalling such as GSK3 and CK1 $\epsilon$  (casein kinase 1 $\epsilon$ ) also compete for binding to axin, and thereby inhibit SAPK/JNK signalling, whereas the binding of APC and  $\beta$  catenin to axin does not interfere with axin mediated SAPK/JNK activation.<sup>44</sup>

## AXIN GENE STRUCTURE, EXPRESSION, AND SUBCELLULAR LOCALISATION

Axin1 (also simply called Axin), which encodes isoforms a and b, and Axin2 (also called Axil or Conductin) have 45% identity at the nucleotide level and the proteins they encode appear to be functionally similar. However, whereas Axin1 is expressed ubiquitously during mouse embryogenesis, Axin2 is expressed in a restricted pattern.<sup>1</sup> Axin1 is the constitutively expressed component of the  $\beta$  catenin degradation complex and is essential for the maintenance of low Wnt signalling activity in the basal state. In contrast, axin2 is upregulated in response to increased  $\beta$  catenin concentrations and thus serves to limit the duration and intensity of the Wnt signal.<sup>47–48</sup> Axin is downregulated in a Wnt dependent manner and is dephosphorylated after Wnt stimulation, which leads to axin1 destabilisation over time. Cells that receive Wnt ligand signals have low concentrations of axin. Biochemical studies show that the intracellular concentrations of axin are approximately 1000 times lower than those of other destruction complex components, suggesting that axin is the limiting factor in this pathway.<sup>49</sup>

### Axin1

Axin1 was first identified as the product of the mouse fused locus. Its human homologue was mapped to chromosome 16p13.3 and shows 87% similarity to the mouse protein.<sup>1</sup> Axin1 “isoform a” encodes a 862 amino acid (aa) polypeptide (GenBank NP\_003493), whereas “isoform b” is a shorter form of axin lacking 36 aa within the N-terminal domain encoded by exon 8 (GenBank NP\_851393) (fig 3A). The function of the polypeptide region encoded by exon 8 is as yet unknown (fig 4A). This splice form is conserved between different species, suggesting a conserved role. The polypeptide encoded by exon 8 is located between the  $\beta$  catenin binding and Dishevelled and axin binding domain (DIX) domains (fig 3A). The region contains a predicted CK1 phosphorylation site and is also close to the axin oligomerisation site, where axin binds to itself. It has been suggested that axin dimerisation is necessary for its stability and function in cells. The spliced exon is also located between two potential axin nuclear export signals.<sup>50</sup>



**Figure 3** Genomic structure of Axin. (A) Axin1 is composed of 10 exons (encoding isoform a). Exon 8 is spliced out in isoform b. (B) Axin2 also consists of 10 exons that encode 843 amino acids (aa) (isoform a) or a 778 aa polypeptide (isoform b). Similar to Axin1, the binding partners of Axin2 are APC (aa 81–200), GSK3 (aa 372–413),  $\beta$  catenin (aa 414–476), and DVL (aa 761–843). So far, all mutations found in Axin2 are located in exon 7. APC, adenomatous polyposis coli; CKI, casein kinase I; DIX, Dishevelled and axin binding domain; DVL, Dishevelled; GSK3, glycogen synthase kinase 3; MEKK1, mitogen activated protein/extracellular regulated kinase kinase 1; NES, nuclear export signal; NLS, nuclear localisation signal; PP2A, protein phosphatase.

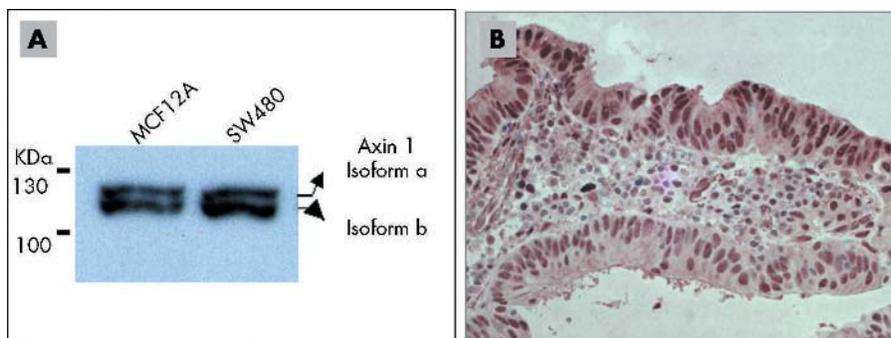
**Axin2**

A homologue of axin, axin2 was identified by virtue of its interaction with  $\beta$  catenin.<sup>16 51 52</sup> Mutation of the Axin2 gene leads to an increase in  $\beta$  catenin concentrations in colorectal cancers with defective mismatch repair systems.<sup>53</sup> Molecular studies revealed that Axin2 contains 10 exons spanning more than 2.5 kb.<sup>54</sup> Similar to Axin1, Axin2 encodes two isoforms (a and b). In isoform b, exon 6 is spliced out, leading to a transcript with 65 fewer aa (S Salahshor, unpublished data, 2004). Fluorescence in situ hybridisation analysis assigned it to human chromosome 17q24, a region that shows frequent loss of heterozygosity in breast cancer, neuroblastoma, and other tumours.<sup>54</sup> Similar to Axin1, Axin2 has binding domains for APC, GSK3, and  $\beta$  catenin (fig 3B).<sup>16</sup>

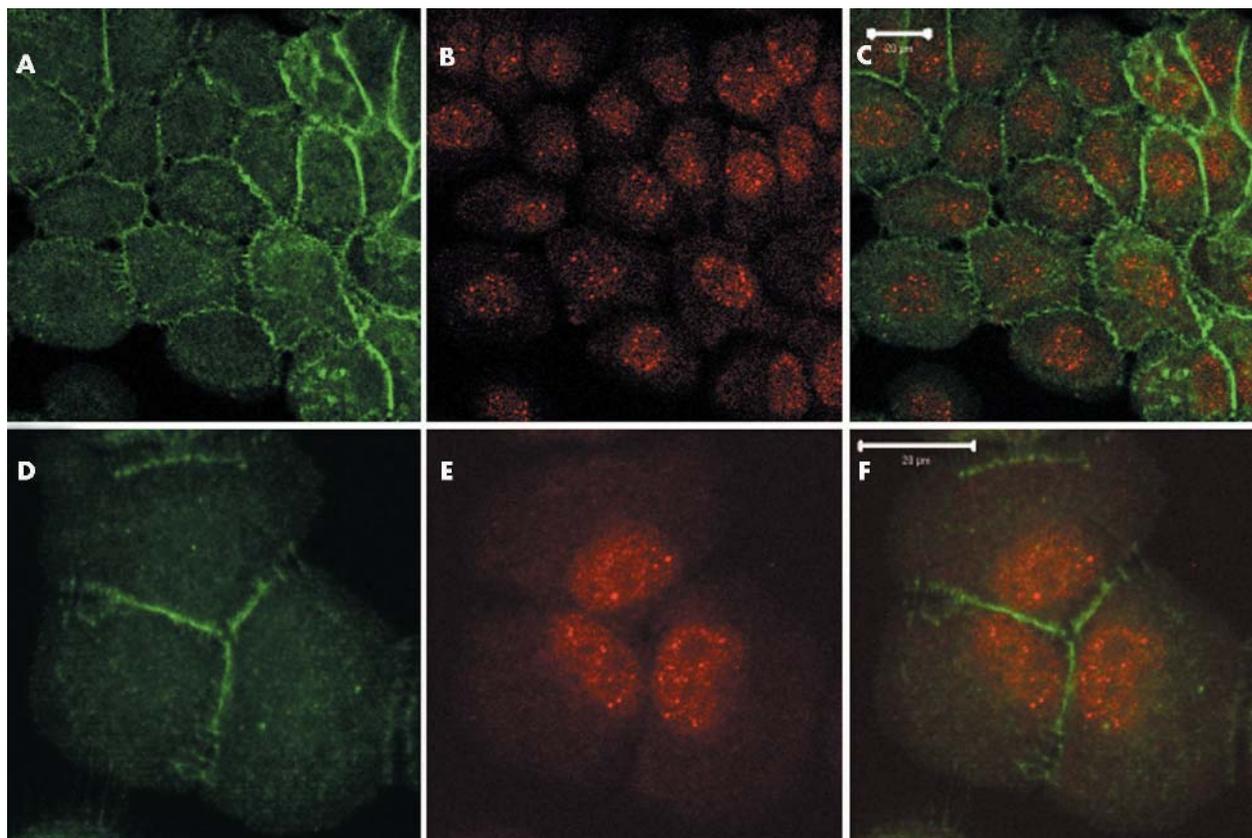
**Subcellular localisation**

Based on the known function and proposed activities of axin, both axins are expected to be located in the cytoplasm of cells where they promote  $\beta$  catenin phosphorylation and degradation. Confocal microscopy of whole mount crypts from patients with colorectal cancer shows that staining for axin1 is diffuse within the nucleus and along lateral cell membranes where  $\beta$  catenin is located, and also in the cytoplasm

where both GSK3 and the cytoplasmic pool of  $\beta$  catenin are found. Cell lysate fractionation of two human epithelial cell lines—HCT116 (colorectal cancer cells with  $\beta$  catenin mutation) and HEK293 (embryonic kidney cells)—reflects the axin localisation pattern seen in tissue. Although axin1 was found mainly in the nucleus of HCT116 cells, in HEK293 cells it was localised in both the nuclear and cytoplasmic compartments.<sup>55</sup> Staining for axin1 is seen in the cytoplasm of normal epithelial cells, whereas it is located in the nucleus in adenocarcinomas and tumours (fig 4B). The subcellular localisation of axin1 appears to be cell type dependent (fig 5). In contrast, axin2 shows strong nuclear staining in normal tissue. Nuclear localisation of axin2 could also be detected in polyps and carcinomas with some cytoplasmic translocation.<sup>55</sup> Despite the similarity between these two molecules, it appears that they are not found within the same compartment of the cell, and thus may have distinct functions. Axin1 can shuttle between the cytoplasm and the nucleus and act as a  $\beta$  catenin chaperone. Two nuclear export (between aa 532 and 667) and three nuclear import signals have been identified on axin1 (fig 3A). Axin1 translocation to the nucleus and its interaction with  $\beta$  catenin is required for axin induced cytoplasmic shifting of  $\beta$  catenin.<sup>50 56</sup>



**Figure 4** Axin expression pattern and subcellular localisation in tumour cell lines and tumour tissue. (A) Western blot analysis of protein extracted from MCF12A and SW480 cells confirmed the existence of two axin1 isoforms (a and b). (B) The subcellular localisation of axin1 was examined in a sporadic colorectal cancer case and axin1 was found to have a predominantly nuclear localisation.



**Figure 5** Subcellular localisation of axin1 in MCF12A cells. Based on the known function of axin1, it is expected to be mainly localised in the cytoplasm. Confocal sections of MCF12A cells stained with (A and D) anti-axin1 and (B and E) anti- $\beta$  catenin antibodies (C and F merged) show both nuclear and cytoplasmic axin1 staining, with a pronounced nuclear localisation, whereas  $\beta$  catenin is located mainly at the cell membrane (scale bars, 20  $\mu$ m). The following antibodies were used: polyclonal anti-axin1 (1/500 dilution; Zymed, South San Francisco, California, USA), monoclonal anti- $\beta$  catenin (1/500 dilution; Signal Transduction Laboratories; Lexington, Kentucky, USA), secondary monoclonal and polyclonal antibodies (1/1000 dilution; Molecular Probes, Eugene, Oregon, USA). Other studies show a more cytoplasmic localisation for axin1. However, the subcellular localisation of axin1 appears to be cell type dependent.

### AXIN BINDING PARTNERS

Both axins contain several domains that mediate direct binding to low density lipoprotein related protein receptor, the Frizzled coreceptor,<sup>57-59</sup> APC (which appears to assist axin in recruiting  $\beta$  catenin to the axin complex),<sup>13</sup> GSK3 $\beta/\alpha$ ,<sup>60</sup>  $\beta$  catenin.<sup>61-62</sup> DVL (DVL-1, DVL-2, and DVL-3),<sup>63</sup> MEKK1, CKI,<sup>64</sup> protein phosphatase 2A (PP2A),<sup>65</sup> frequently rearranged in advanced T cell lymphomas (Frat1),<sup>66</sup> and a homodimerisation domain (figs 3 and 6).<sup>67</sup>

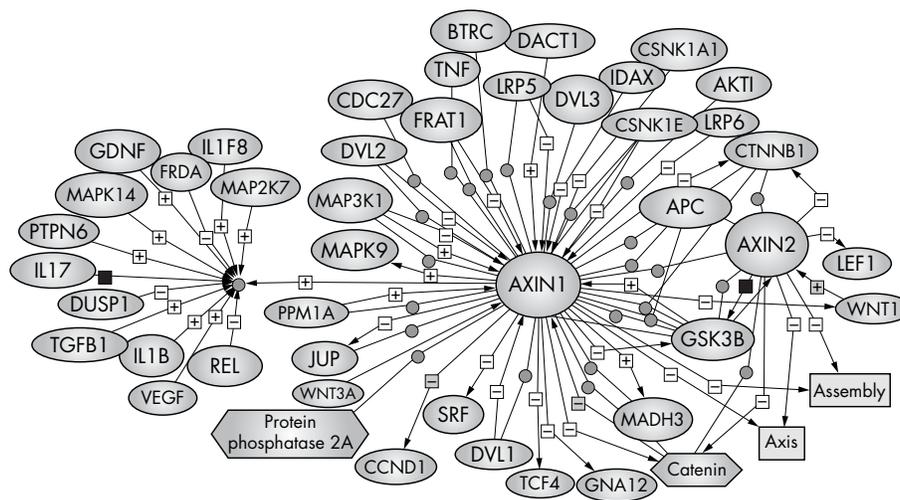
### APC

The region of axin involved in APC binding shows significant homology to members of the regulators of G protein signalling (RGS) family.<sup>1-12</sup> The region of APC that interacts with axin consists of a conserved sequence of approximately 20 aa containing a Ser-Ala-Met-Pro (SAMP) motif.<sup>16</sup> The APC gene contains three SAMP repeat sequences, all of which are located after its mutation cluster region, where most truncating mutations in cancer are found. These mutations eliminate all axin binding sites on APC, but preserve some of the  $\beta$  catenin binding domains, indicating the important role that the interaction between axin and APC plays in APC tumour suppressor activity.<sup>22-68</sup> Thus, the tumorigenic potential of mutated APC correlates with loss of binding to axin1/axin2, rather than loss of  $\beta$  catenin binding. APC binding to axin via its RGS domain is required for the efficient downregulation of  $\beta$  catenin by APC.<sup>22-51</sup> However, overexpression of axin in colorectal cancer cell lines bearing a mutated form of APC that lacks an axin binding domain can

still promote  $\beta$  catenin downregulation.<sup>16-61-69</sup> Similarly, axin mutants lacking the APC binding domain are still capable of  $\beta$  catenin downregulation when overexpressed.<sup>51-62</sup> These results suggest that APC might be only a cofactor for axin1/axin2. However, multiple binding sites on APC for  $\beta$  catenin (10 binding sites) and axin1/axin2 (three binding sites) may be important for the efficient assembly of the  $\beta$  catenin degradation machinery in different cell types or various *in vivo* conditions. These results further indicate that the concentration of axin rather than its interaction with other components of Wnt signalling might control its activity in cells, and that axin is the rate limiting factor in these reactions. In contrast, in SW480 colorectal cancer cell lines, which carry a truncated form of APC,  $\beta$  catenin is stabilised. Expression of ectopic APC leads to  $\beta$  catenin degradation.<sup>22-70</sup> Endogenous axin1 and axin2 are present in this cell line, but appear not be capable of downregulating  $\beta$  catenin in the absence of wild-type APC.

“Direct binding of APC to  $\beta$  catenin is not essential for APC to degrade  $\beta$  catenin, whereas the binding of axin to  $\beta$  catenin is necessary for it to degrade  $\beta$  catenin”

However, overexpression of exogenous axin2 can induce  $\beta$  catenin degradation in SW480 colon carcinoma cells. Overexpression of an axin2 mutant that lacks the  $\beta$  catenin binding domain does not induce  $\beta$  catenin degradation, whereas an axin2 mutant lacking the RGS domain (APC



**Figure 6** Axin regulators and binding partners. To bring all the relevant information about axin together, we have used PathwayAssist software (Stratagene) to generate a network of molecules that are either positively (+) or negatively (-) regulated by axin. Each interaction is annotated with the source references and contains hyperlinks to the source literature on PubMed. The biological data have been extracted from PubMed. The references that support the interaction or regulation for each molecule can be viewed by holding the mouse on the plus or minus sign. This pathway is an online navigation tool ([http://kinase.uhnres.utoronto.ca/Sima/axins\\_pathway/](http://kinase.uhnres.utoronto.ca/Sima/axins_pathway/)).

binding domain) can induce degradation.<sup>16</sup> A  $\beta$  catenin mutant that does not interact with axin2 is resistant to degradation induced by the ectopic expression of axin2 in SW480 cells. However, these  $\beta$  catenin mutants were efficiently degraded when wild-type APC was introduced.<sup>71</sup> These observations indicate that direct binding of APC to  $\beta$  catenin is not essential for APC to degrade  $\beta$  catenin, whereas the binding of axin2 to  $\beta$  catenin is necessary for it to degrade  $\beta$  catenin.

### $\beta$ Catenin

Axin1 has been shown to interact directly with  $\beta$  catenin at residues adjacent to the GSK3 $\alpha/\beta$  binding domain,<sup>12</sup> and  $\beta$  catenin binding to axin2 is abolished or strongly reduced by mutations in  $\beta$  catenin ARM (armadillo repeat) 3 and 4.<sup>71</sup> A direct interaction between axin2 and  $\beta$  catenin in the same region as axin1 has been shown. This interaction promotes the phosphorylation of  $\beta$  catenin by CKI $\alpha$  and GSK3, which is required for the ubiquitination and subsequent degradation of  $\beta$  catenin. The degradation of  $\beta$  catenin can also occur in a GSK3/CKI $\alpha$  phosphorylation independent manner. Siab (mammalian homologue of drosophila gene sina) binds to the ubiquitin conjugating enzyme through its N-terminal region and forms a complex with Ebi. Ebi is an F-box protein that binds to  $\beta$  catenin and forms a complex that functions as a ubiquitin ligase and is able to downregulate  $\beta$  catenin in a p53 inducible manner.<sup>72-73</sup> Recently, it was shown that activated p53 can downregulate  $\beta$  catenin. It was suggested that p53 induces the faster mobilisation of axin into the  $\beta$  catenin degradation complex, and thereby stimulates  $\beta$  catenin degradation.<sup>74</sup> These observations imply that axin might be able to promote the degradation of  $\beta$  catenin, through both phosphorylation dependent and independent pathways.

### GSK3

GSK3 $\alpha$  and GSK3 $\beta$  are essential players in the  $\beta$  catenin destruction complex. GSK3 $\alpha/\beta$  are serine/threonine kinases that phosphorylate three conserved serine and threonine residues in the N-terminal domain of  $\beta$  catenin.<sup>75-76</sup>  $\beta$  Catenin is a poor substrate of GSK3 in vitro, and GSK3 does not bind  $\beta$  catenin directly, requiring axin and APC to facilitate its interaction with the  $\beta$  catenin target.<sup>1-77-78</sup> Axin1 dramatically (> 20 000 times) facilitates the phosphorylation of APC and  $\beta$  catenin by GSK3 in vitro.<sup>61</sup> Phosphorylation by GSK3 $\alpha/\beta$  requires a priming phosphorylation by a third party kinase. In the case of  $\beta$  catenin, this priming enzyme is CKI $\alpha$ .<sup>79</sup> Axin

itself is phosphorylated by GSK3 $\alpha/\beta$  and this modification increases its stability.<sup>80</sup> Unphosphorylated axin has a lower affinity for  $\beta$  catenin, reducing its ability to promote the formation of the  $\beta$  catenin destruction complex.<sup>27-80-81</sup> Axin1 contains several possible GSK3 $\alpha/\beta$  phosphorylation sites, and mutation of these leads to reduced axin phosphorylation by GSK3.

“Axin1 dramatically facilitates the phosphorylation of APC and  $\beta$  catenin by glycogen synthase kinase 3 in vitro”

In drosophila, expression of a hypomorphic allele of Armadillo (the fly homologue of  $\beta$  catenin) on a background lacking Zeste-White3/Shaggy (the fly homologue of GSK3), revealed that the concentration of  $\beta$  catenin/Armadillo was still sensitive to expression of Wingless, the fly homologue of Wnts. This effect was mediated by changes in the concentration of axin.<sup>59-82</sup> As mentioned previously, axin concentrations in cells are the lowest of all of the regulatory components and could therefore be rate limiting.<sup>49</sup> However, Wnt signalling in mammalian cells does not appear to alter axin1 protein concentrations (B Doble and J Woodgett, unpublished observation, 2004). These data suggest that the Wnt pathway may use multiple mechanisms to regulate the concentration of  $\beta$  catenin, although the dominant mechanism appears to be via phosphorylation.

### DVL

DVL binds to the C-terminal region of axin, which includes the DIX domain, and inhibits axin activation.<sup>83</sup> The association of DVL with axin modulates the ability of axin to dimerise. DVL also binds CKI, which can promote Wnt3a mediated DVL phosphorylation. Phosphorylated DVL has a high affinity for Frat, which binds to and inhibits GSK3.<sup>63-84</sup> In Wnt stimulated cells, Frat bound to DVL might be able to prevent GSK3 bound to axin from phosphorylating  $\beta$  catenin (fig 3A). Both DVL and axin have DIX domains that are necessary for the binding of DVL and axin to intracellular vesicles and actin filaments, which suggests that axin and DVL may regulate receptor mediated endocytosis of the Wnt signalling pathway.<sup>85</sup> DVL also plays a role in relocating axin to the plasma membrane upon Wnt signalling.<sup>86</sup> Studies on living embryos have confirmed previous findings that Wnt signals cause a relocalisation of axin from the cytoplasm to the plasma membrane and that this relocalisation is DVL dependent.<sup>59</sup>

**Table 1** Sequence variants of the Axin1 gene found in human cancer

Tissue type	Exon/nt or aa	Position (aa)	Domain(s) affected	Ref
CRC	1	S215L	APC-MEKK1?	94
CRC	1	R103M	APC	95
CRC	1	L101P	APC	95
CRC	3	R353H	GSK3	95
CRC	1	T122A	APC	95
CRC	4	E411D	GSK3	95
CRC	4	M418I	GSK3	95
CRC	4	R395C	GSK3	95
CRC	5	G584S	$\beta$ Catenin	95
CRC	2	Y305X	MEKK1	96
CRC	1	K203M	APC	96
CRC	2	N307K	MEKK1	96
CRC	4	H394N	GSK3	96
CRC	10	P848L	Axin-DVL	96
CRC	10	E852G	Axin-DVL	96
CRC	1	S215L	APC-MEKK1?	94
MB	1-5 (aa 89-528)	Del	APC-GSK3- $\beta$ catenin	21
MB	1-5 (aa 111-571)	Del	APC-GSK3- $\beta$ catenin	21
MB	1-5 (aa 59-532)	Del	APC-GSK3- $\beta$ catenin	21
MB	9-10	Del	Axin-DVL	21
MB	8-10	Del	Axin-DVL	21
MB	6-10	Del	PP2A-axin-DIX	21
MB	7-10	Del	PP2A-axin-DIX	21
MB	1	P255S	APC	21
MB	1	P255S	MEKK1	20
MB	1	S263C	MEKK1	20
HCC	1	Stop	APC-DVL-axin	2
HCC	4	Stop	GSK3-DVL-axin	2
HCC	4	5 bp del	$\beta$ Catenin	2
HCC	5	13 bp del	$\beta$ Catenin	2
HCC	3	P345L	MEKK1	97
HCC	1	L106R	APC	97
HCC	2 (1076 del 1 bp)	Frameshift	MEKK1?	97
HCC	5	Ins QVHH	$\beta$ Catenin-PP2A?	97
HCC	5	G425S	$\beta$ Catenin	97
HB	10	R842Q	Axin-DIX	97
HB	6	G651S	PP2A?	97
HB	2	T95M	APC	98
EM	4	A397V	GSK3	99
SCC	4	G419A	GSK3	100
OEA	4	L416X	GSK3	101
ACC	7	P661L	PP2A	102
ACC	5	G433E	$\beta$ Catenin	102
ACC	10	E842K	Axin-DVL	102
ACC	10	F824K	Axin-DVL	102
ACC	6	S628Y	PP2A?	102

Mutations found in the axin1 coding sequence are scattered throughout the whole gene. There are some differences between the alterations found in different forms of tumours. Large deletions are more common in medulloblastomas, whereas missense mutations occur more frequently in hepatocellular carcinomas. Silent mutations and polymorphisms reported in axin1 are not included in this list. Protein and mRNA sequences are based on Swissprot and GenBank accession numbers O15169 and AF009674, respectively.

aa, amino acids; ACC, adenoid cystic carcinoma; APC, adenomatous polyposis coli; bp, base pair; CRC, colorectal cancer; del, deletion; DVL, Dishevelled; EM, endometrial cancer; GSK3, glycogen synthase kinase 3; HB, hepatoblastoma; HCC, hepatocellular cancer; MB, medulloblastomas; MEKK1, mitogen activated protein/extracellular regulated kinase kinase kinase 1; nt, nucleotide; OEA, ovarian endometrioid adenocarcinoma; PP2A, protein phosphatase 2A; SCC, oesophageal squamous cell carcinoma.

## CKI/II

CKI has recently been found to be an activator of Wnt signalling because microinjection of CKI into xenopus induces a secondary axis.<sup>87</sup> The overexpression of CKI mimics Wnt signalling by stabilising  $\beta$  catenin and thereby inducing the expression of  $\beta$  catenin target genes. CKI has been detected in the axin-GSK3 complex and appears to phosphorylate APC and  $\beta$  catenin (at Ser45) in an axin dependent manner.<sup>64 79 88</sup> CKI also competes for MEKK1 binding and can attenuate axin-SAPK/JNK signalling.<sup>89</sup> In contrast, although CKII also interacts with axin, it does not have an inhibitory effect on SAPK/JNK signalling.<sup>89</sup> CKII interacts and phosphorylates DVL, which is thought to inhibit the function of axin, thereby stimulating Wnt signal transduction.<sup>90</sup>

## PP2A

PP2A is a broad specificity serine/threonine protein phosphatase that comprises a catalytic "C" subunit and various

"A" and "B" regulatory subunits that confer selective binding to proteins and scaffolds within cells. PP2A physically interacts with axin and APC in the axin-GSK3- $\beta$  catenin destruction complex and decreases the phosphorylation of axin, which suggests a positive role for PP2A in Wnt signal transduction.<sup>14</sup> Phosphorylation of APC is also important for  $\beta$  catenin binding and its subsequent degradation. PP2A has been implicated as a positive effector for Wnt mediated signalling.<sup>91</sup> However, the phosphatase has other, confounding effects, probably because of its action on both positive and negative effectors of the pathway, such that its role is clearly complex.<sup>14 65 92 93</sup>

## AXIN VARIANTS IN CANCER

Alterations in both axin1 and axin2 have been detected in several different tumours (tables 1 and 2). Mutations are found in most axin domains including the APC (RGS) and  $\beta$  catenin binding domains. Axin sequence variants have also

**Table 2** Axin2 variants in human diseases

Tissue type	Exon/nt (bp)	Position (aa)	Domain affected	Ref
CRC	7	L688X	?	53
CRC	7	E706X	?	53
OEA	7	G665X	?	101
CRC	7	E706Ter	?	103
EM	7	S658C	?	99
CRC/ Oligodentia	7	R656X	?	104
CRC/ Oligodentia	7 (1995 ins G)	?706X	?	104
HCC	7	R659W	?	97
HCC	7 (2102; del 12 bp)	del TTPR	?	97

Approximately 20% of mismatch repair deficient colorectal cancers harbour mutations in axin2. Most mutations are located around a G<sub>7</sub> repeat in exon 7 (nt 637–714), which results in premature protein truncation.  
 aa, amino acids; APC, adenomatous polyposis coli; bp, base pair; CRC, colorectal cancer; del, deletion; EM, endometrial cancer; GSK3, glycogen synthase kinase 3; HB, hepatoblastoma; HCC, hepatocellular cancer; ins, insertion; nt, nucleotide; OEA, ovarian endometrioid adenocarcinoma.

been found in colon, ovarian, endometrioid, adenocarcinoma, and HCC cell lines (table 3). Biochemical and functional studies have shown that these mutations interfere with the binding of GSK3 and that they also alter the interaction between axin and two upstream activators of TCF dependent transcription, Frat1 and DVL.<sup>94</sup> Several studies have investigated the role of axin in several types of tumour.

**Medulloblastoma**

Medulloblastoma (MB) is the most common malignant brain tumour in children.<sup>105</sup> Most MBs are sporadic; however, patients with germline mutations in APC or PTCH (patched) genes carry a higher risk of this disease.<sup>106</sup> In addition, mutations in β catenin, another component in the Wnt pathway, have been detected in MB tumours.<sup>107–109</sup> Recently, several deletions and single somatic point mutations in Axin1 have been found in sporadic MB cases.<sup>20 21 110</sup> These aberrations include somatic point mutations, deletions, and loss of heterozygosity. The C-terminal deletion in these cases corresponded to loss of exons 6–10, where the DIX and oligomerisation domains are located. Oligomerisation of Axin1 is crucial for the inhibition of TCF transcription,<sup>67</sup> and C-terminal deleted axin1 fails to downregulate β catenin and cell growth in the SW480 colon cancer cell line.<sup>63</sup> Another type of inframe deletion that removes exons 1–5, the APC binding domain, has also been found in MB. The truncated protein lacks binding sites for APC, GSK3, and β catenin (table 1). This mutant is also incapable of downregulating β catenin. In addition, some truncated forms of axin1 may act as dominant negatives and inactivate endogenous axin1.<sup>111</sup>

**Table 3** Axin1 variants identified in cancer cell lines

Cell line	Type	Exon	Position (aa)	Domain(s) affected	Ref
MDAH-2774	OEA	5	V555I	?	101
SNU475	HCC	1–2	Del	APC	2
SNU423	HCC	3	13 bp del	GSK3	2
Alexander	HCC	4	Del	GSK3	2
HCT-8	CRC	4	L396M	GSK3	94

aa, amino acids; APC, adenomatous polyposis coli; bp, base pair; CRC, colorectal cancer; del, deletion; GSK3, glycogen synthase kinase 3; HCC, hepatocellular cancer; OEA, ovarian endometrioid adenocarcinoma.

**Colorectal cancer and familial tooth agenesis**

Many components of the Wnt signalling system are mutated in colorectal cancer. Germline loss of function mutations in the APC gene are associated with an inherited form of colorectal cancer—familial adenomatous polyposis—with 90–95% penetrance. Somatic APC mutations are also found in most sporadic colorectal cancers.<sup>112</sup> Alterations in other components of Wnt signalling, including β catenin, TCF, axin1, and axin2, found in colorectal cancer indicate the important role that this pathway plays in the aetiology of this disease.<sup>113</sup> Most Axin1 mutations in colorectal cancer occur between exon 1 and 5, where the APC, GSK3, and β catenin binding domains are located (table 1). Mutations in axin2 have been found in approximately 20% of mismatch repair deficient colorectal tumours (table 2).<sup>6 53 94 101 103</sup> In most cases, one base deletion or insertion occurs in the mononucleotide repeat sequences located in exon 7, leading to a frame shift and premature protein truncation.<sup>53</sup> These mutations lead to elimination of the DIX domain, where DVL binds and negatively regulates axin activity. This domain is also essential for homo-oligomerisation of axin. The mutant form of axin2 appears to be more stable than the wild-type protein. Transfection of normal fibroblasts with axin2 mutants led to the accumulation of β catenin in the nuclei.

“Alterations in other components of Wnt signalling, including β catenin, TCF, axin1, and axin2, found in colorectal cancer indicate the important role that this pathway plays in the aetiology of this disease”

Immunohistochemical staining of β catenin in tumours with defects in axin2 also showed nuclear accumulation of β catenin in the cells. These results suggest a dominant negative effect of axin2 mutant proteins in cells. High levels of Axin2 gene expression have been detected in most human colon cancer cell lines (SW480, SW620, LoVo, SW620, Caco2, HT29, HCT116, T84, HCT15, and Alab), in addition to colorectal tumours. Axin2 appears to be a transcriptional target of the Wnt signalling pathway. Upregulation of Axin2 by Wnt–β catenin appears to constitute a negative feedback loop that acts to restrain or desensitise Wnt signalling. Axin2 gene expression increases in response to raised concentrations of β catenin, thereby modulating the duration and activity of the Wnt signal.<sup>47 48</sup> In addition to colorectal cancer, several Axin2 mutations have been found in patients with oligodentia. Linkage analysis of a Finnish family with colorectal cancer in association with oligodentia revealed a high logarithm of the odds score in chromosome 17 between markers D17S949 and D17S1352, where more than 80 known or predicted genes (including Axin2) are located. Direct mutational analysis of the coding sequence of Axin2 identified a base pair transition in exon 7, leading to premature termination of translation of Axin2, whereas family members with normal dentition showed no sign of Axin2 mutation or neoplasia. Axin2 is expressed in developing dental tissues, and germline Axin2 mutations found in patients with oligodentia provided the first evidence for its function.<sup>104</sup> In a form of familial colorectal cancer, Gardner syndrome, the occurrence of odontomas and supernumerary teeth in association with familial adenomatous polyposis has been reported.<sup>114–117</sup> These results indicate that overactivation of Wnt signalling as a result of mutation of a component of the β catenin destruction complex, in addition to carcinogenesis, may lead to the failure of tooth development (tables 1–3).

**Hepatocellular carcinomas**

HCC and hepatoblastoma (HB) are primary liver cancers that occur predominantly in adults and children, respectively.

Alteration of  $\beta$  catenin occurs in approximately 20% of HCCs and 40–89% of HBs.<sup>23 118–121</sup> A fraction of HCCs (~ 10%) with wild-type  $\beta$  catenin have mutations in Axin1 or Axin2 instead.<sup>2 122</sup> These data suggest that alterations in the Wnt pathway are involved in the pathogenesis of a large proportion of both HBs and HCCs. The axin1 mutations identified in the previous reports include truncation mutations as a result of either small deletion/insertion or nonsense mutations, although Axin1 mutations found in liver cancer show a different spectrum of alterations. Most of the mutations found in these cases were predominantly missense. These Axin mutations apparently only affect one allele, so that their oncogenic effect may result from the dominant negative activity of the mutant Axin, which causes stabilisation of free  $\beta$  catenin.<sup>16</sup> In addition, detection of loss of heterozygosity at the Axin1 locus suggests that axin1 functions as a tumour suppressor. However, in a subset of tumours mutations in Axin1 or Axin2 were found in addition to mutations in  $\beta$  catenin.<sup>97</sup> The expression of wild-type Axin1 in HCC or colorectal cancer cells with APC,  $\beta$  catenin, or Axin1 mutations led to the induction of apoptosis.<sup>2</sup> Even though axin1 and axin2 are both able to downregulate  $\beta$  catenin when overexpressed, and show similar biochemical characteristics, axin2 does not compensate for axin1 mutations in HCCs. Therefore, axin1 and axin2 expression is either cell type dependent or, more likely, these two proteins are not functionally equivalent.

#### Ovarian endometrial adenocarcinomas

Ovarian cancer is one of the most frequent gynaecological malignancies in women.<sup>123</sup> A subtype of ovarian cancer, endometriosis, often shows defects in the components of the Wnt signalling pathway. Although mutations in APC are rare in this type of tumour, mutation of  $\beta$  catenin is found in 16–24% of cases.<sup>24 124 125</sup> Recently, mutations in axin1 and axin2 have been detected in a subset of ovarian endometrial adenocarcinomas, where activation of TCF dependent transcription could be detected in a tumour with an Axin2 frameshift mutation (tables 1 and 2). As noted in cell lines, the mutated form of axin2 appears to be more stable than the wild-type axin2 protein.<sup>101</sup>

#### Oesophageal squamous cell carcinoma

Oesophageal cancer accounts for 7% of all gastrointestinal malignancies, but in some regions of Asia its incidence may be as high as 170/100 000 of the population. About half of the oesophageal cancers diagnosed are squamous cell carcinomas (SCCs). Several studies suggest a role for defects in Wnt signalling in the pathogenesis of SCC. Deletion of chromosome 17 (where Axin2 is located) has been found in 45% of SCCs.<sup>126</sup> Immunohistochemical studies of axin1 have shown cytoplasmic localisation of the protein in normal stratified squamous epithelium of the oesophagus, whereas a mixed pattern of expression was detected in tumours. Protein expression analysis of seven oesophageal SCC cell lines revealed differential axin expression. Four cell lines (TE1, TE15, TT, and TTn) showed high expression, whereas another three cell lines (TE2, TE8 and TE13) showed very low axin1 expression. However, northern blot analysis of the same cells showed similar amounts of RNA in all cases. Several mutations and polymorphisms have been reported in SCC tumours and cell lines (tables 1 and 3), and a correlation between reduced axin1 expression and tumour progression has been suggested in oesophageal SCC.<sup>100 127</sup>

#### Roles of axin/GSK3 in Alzheimer's disease

In addition to tumorigenesis, defects in the Wnt signalling pathway have also been postulated to contribute to the pathogenesis of Alzheimer's disease (AD).<sup>128–130</sup> The pathology of this form of dementia involves the deposition of amyloid

plaques, which are made up of A $\beta$  peptides derived from the abnormal cleavage of amyloid precursor protein, and neurofibrillary tangles, which are composed of hyperphosphorylated forms of the microtubule associated protein, tau. These two lesions are associated with neuronal cell death. GSK3 is one of several protein kinases that phosphorylates tau at sites observed in the neurofibrillary tangles.<sup>131–133</sup> Axin negatively affects tau phosphorylation by GSK3.<sup>134</sup> Tau that has been phosphorylated by GSK3 has a decreased capacity to bind and stabilise microtubules.<sup>135</sup> Altered expression of PP2A, another protein implicated in Wnt signalling, has also been associated with AD pathology and appears to play a major role in regulating tau phosphorylation in the adult brain.<sup>136–139</sup> These results have led to the hypothesis that deregulation of GSK3 might be involved in the pathogenesis of AD.<sup>140 141</sup> Considering the central role that the scaffolding protein axin plays in regulating GSK3 mediated phosphorylation of  $\beta$  catenin, and the fact that axin negatively regulates GSK3 mediated tau phosphorylation, it has been suggested that deregulation of the phosphorylation status of GSK3 may contribute to tau pathology in AD. A direct role for Wnt signalling in AD pathology remains to be demonstrated. However, it can be speculated that events leading to a decrease in GSK3–axin complex formation, and ultimately axin concentrations, may allow the aberrant phosphorylation of tau by GSK3 at primed sites that are increased in the AD brain, and thus contribute to tau dysfunction and pathology.<sup>142</sup>

#### AXIN: A POTENTIAL TARGET FOR CANCER TREATMENT

Given the clear link between defects in Wnt signalling components and several human cancers, compounds that either positively or negatively regulate these molecules according to their role in the pathway will provide valuable reagents for the development of anticancer drugs. Because axin positively regulates the SAPK/JNK (apoptosis) pathway and negatively regulates the Wnt (survival) pathway, and is the limiting factor in these systems, a search for axin antagonists or agonists might lead to the discovery of compounds that have potential for the treatment of cancer. To ascertain whether the Axin mutations found in cancers were biologically important, normal Axin1 genes were inserted into the cancer cells and their effects on cell growth monitored. In the case of HCC cell lines, the introduction of Axin appeared to induce programmed cell death, suggesting a therapeutic potential. Although this opens up the possibility of using Axin as a cargo gene in gene therapy, small molecule approaches are also being pursued.

“A search for axin antagonists or agonists might lead to the discovery of compounds that have potential for the treatment of cancer”

The high frequency of mutations in the Wnt pathway in specific tumour types suggests that the activated transcriptional output of these pathways efficiently misregulates multiple growth regulatory functions. Because axin can degrade  $\beta$  catenin when overexpressed in cells that lack functional APC it is a good candidate for cancer treatment. One drawback is that although the overexpression of axin1 strongly promotes the downregulation of wild-type  $\beta$  catenin in colon cancer cells, mutant, oncogenic  $\beta$  catenin is unaffected.

Thus, Axin treatments would be selective for APC associated cancers. More studies of the mechanistic details of these molecules are required to elucidate the precise role and effect of axin stimulation in cancer treatment.

### Take home messages

- Axins (both Axin1 and its homologue Axin2) are tumour suppressor genes
- Deregulation of axin has been found in different forms of cancer
- Wnt signals modulate axin concentrations in the cell
- Axin is a rate limiting component of different signalling pathways
- The Wnt, stress activated protein kinase/Jun N-terminal kinase, and transforming growth factor  $\beta$  signalling pathways are regulated by axin
- Upregulation of axin leads to the phosphorylation and degradation of  $\beta$  catenin
- The overexpression of axin promotes apoptosis

### CONCLUDING REMARKS

A large body of evidence indicates that the regulation of  $\beta$  catenin stability and concentration in cells by the axin–APC–GSK3 complex is crucial for both embryogenesis and carcinogenesis. Axins act as negative regulators for Wnt signalling, and positive regulators for SAPK/JNK signalling. Because axin itself is sensitive to Wnt signals, axin concentrations in cells fluctuate, which probably impacts upon its other functions. Despite being discovered in 1997, there is much to be learned about the physiological functions and roles of axin. In common with several other molecules in the Wnt signalling pathways, axin binds to different components and regulates opposite functions in the cell. The question is how axin operates to maintain the specificity of these different signalling pathways. Downregulation of axin can be just as catastrophic as too much activity. Axin2 overexpression in cells leads to apoptosis, yet upregulation of axin2 is found in most colorectal cancers. Deregulation of axin2 can be detected in adenomas where cells do not show  $\beta$  catenin nuclear accumulation, which indicates that axin2 might be an early marker of tumour initiation. Furthermore, axin2 is mostly located in the nucleus, and has access to the “active” form of its target molecule,  $\beta$  catenin. It remains to be determined how the basal concentrations of axins are controlled and how their nuclear and cytoplasmic localisation is accomplished. Currently, our understanding of axins suggests that these proteins play key assembly functions for promoting efficient protein–protein interaction and stand at the crossroads of several signalling paths—helping to integrate and coordinate the plethora of continuously changing signals.

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### APPENDIX 1 ONLINE LINKS AND DATABASES

- [http://kinase.uhnres.utoronto.ca/Sima/axins\\_pathway/](http://kinase.uhnres.utoronto.ca/Sima/axins_pathway/) (Axin page)
- <http://www.stanford.edu/~rnusse/wntwindow.html> (Wnt page)
- <http://us.expasy.org/prosite/> (Prosite database)

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# PostScript

## CORRESPONDENCE

### Poorly differentiated hepatocellular carcinoma with unusual tubular structures

The patient was a 70 year old woman. A tumour in liver segment 8 arose in a background of cirrhotic liver with chronic hepatitis C and reached a size of 6.0 cm in six months. The patient's serum concentration was raised (17101 ng/ml), and the tumour was suspected to be hepatocellular carcinoma (HCC) based on various image findings. An extended liver anterior segmentectomy was performed, and serum  $\alpha$  fetoprotein returned to normal immediately after surgery.

Although the macroscopic findings were compatible with conventional HCC (fig 1A), the histology of the tumour was atypical—the tumour cells mainly formed irregular tubular structures filled with a bloody/serous or bloody fluid (fig 1B), and small tubular or acinar-like structures were also found (fig 1C). Solid structures were seen in a small portion of the tumour (fig 1D), and massive bleeding was also seen. The tumour cells had abundant eosinophilic granular cytoplasm and round nuclei with moderate variations in size and shape. The typical trabecular pattern was not seen, and no evidence of desmoplastic stroma, extracapsular proliferation, vascular invasion, or Alcian blue/periodic acid Schiff positive mucin was

seen. In addition, a typical moderately differentiated HCC (measuring 1.0 cm) with trabecular pattern was also found.

Immunohistochemical examination revealed that the tumour cells showed diffuse and strong reactivity for vimentin and pankeratin (AE1/3), focal reactivity for  $\alpha$  fetoprotein and HepPar 1, and negativity for calretinin, Wilms' tumour 1 protein, c-kit, CD34, cytokeratin 7, cytokeratin 19, cytokeratin 20, low molecular weight cytokeratin (CAM5.2), epithelial membrane antigen, chromogranin A, synaptophysin, neurone specific enolase, carcinoembryonic antigen, CA125, 2A2, 2G10, and 4C4. The tumour cells had a high proliferative activity, scoring 60% on the MIB-1 labelling index.

All candidate tumour types with the exception of HCC (cholangiocellular carcinomas, metastatic adenocarcinomas, primary malignant mesotheliomas, carcinoid tumours, and germ cell tumours) were ruled out clinically and histologically. Pseudoglandular formation is a common histological manifestation of HCC, and pelioid-type HCC shows large vascular lakes within the tumour, mimicking peliosis hepatis. Therefore, we consider this tumour to resemble such types of HCC.

Recently, intermediate liver carcinomas and hepatic stem cell malignancies have been reported.<sup>1,2</sup> However, an apparent stem cell component was not prominent in the present tumour, and the negativity for c-kit, the hypochromatic nuclei, and the absence of

desmoplastic stroma were not compatible with these types of tumours.

The reticular-like pattern suggested a yolk sac tumour, and an association between hepatitis C virus infection and yolk sac tumours has been suggested.<sup>3</sup> However, specific features, such as Schiller-Duval bodies, a cystic pattern, and hyaline globules, were not detected. In addition, the tumour was immunohistochemically negative for 2A2, 2G10, and 4C4, which have been reported to be specific to yolk sac tumours.

A strong reactivity for vimentin is associated with metastatic HCCs or sarcomatous HCCs,<sup>4,5</sup> indicating a highly malignant form of HCC. Clinically, this tumour showed rapid growth and a high proliferative activity of 60% as assessed by the MIB-1 labelling index.

Considering the various findings described above, we finally diagnosed this tumour as an unusual type of HCC with poorly differentiated features presenting with a high degree of malignancy. Thirteen months after surgery, a new tumour was detected in liver segment 2 and percutaneous ethanol injection therapy was performed.

J Yamaguchi

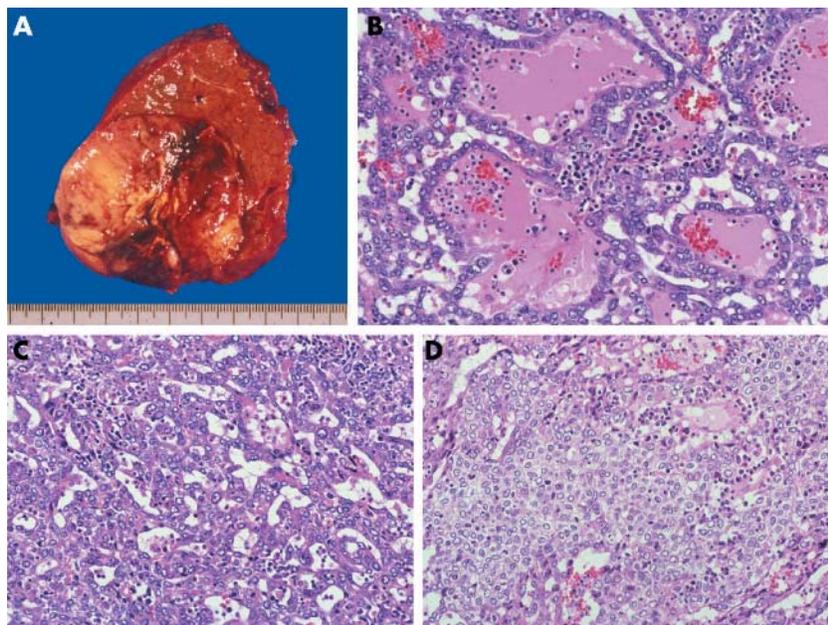
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**Figure 1** (A) Macroscopically, the nodular S8 tumour has a fibrous capsule. The cut surface of the tumour is well circumscribed, expanded, and yellow/white to dark red in colour. (B) The tumour cells have abundant eosinophilic granular cytoplasm and round nuclei with moderate variations in size and shape. The irregular trabecular structures are filled with a bloody/serous or bloody fluid without mucin production and desmoplastic tissue. The typical trabecular pattern was not seen (haematoxylin and eosin (H&E) stain, original magnification,  $\times 200$ ). (C) Small tubular or acinar-like patterns are also visible (H&E stain; original magnification,  $\times 200$ ). (D) Solid structures are seen in a small portion of the tumour (H&E stain; original magnification,  $\times 200$ ).

The patient gave informed consent for this letter to be published.

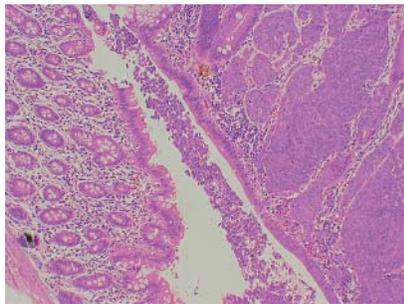
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## Metastasis of a caecal neuroendocrine carcinoma to the thyroid gland

Metastatic tumours to the thyroid have been reported to arise from several organs.<sup>1,2</sup> We describe a unique case of caecal neuroendocrine carcinoma (NEC) metastatic to the thyroid gland, mimicking a primary medullary thyroid carcinoma (MTC).

A 56 year old woman was referred after complaining of dysphagia and hoarseness.

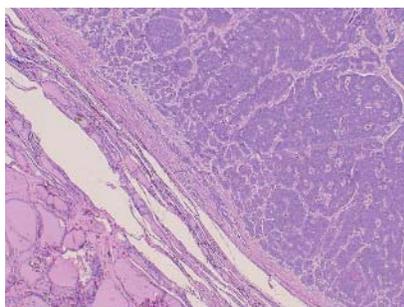


**Figure 1** Primary neuroendocrine carcinoma of the caecum. Haematoxylin and eosin stain; original magnification,  $\times 10$ .

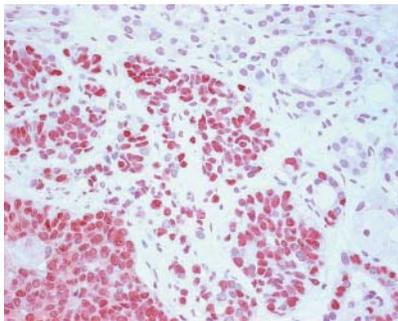
Fifteen months before, she underwent surgery because of a well differentiated caecal NEC, low grade malignant, with metastases to the left ovary, the omentum, and the abdominal lymph nodes (World Health Organisation classification).<sup>3</sup> The tumour was composed of spindle shaped cells, exhibiting scanty eosinophilic cytoplasm, salt and pepper nuclei, and inconspicuous nucleoli (fig 1). Neoplastic cells showed intense reactivity with antibodies against CAM 5.2, AE1/AE3, cytokeratin 7, cdx-2, chromogranin A, synaptophysin, serotonin, and neurone specific enolase; there was weak reactivity for calcitonin and carcinoembryonic antigen. In contrast, no immunoreactivity was detected for thyroid transcription factor 1 or vimentin.

On examination, a firm nodule was felt in the left lobe of the patient's thyroid gland; attempts at fine needle aspiration biopsy did not yield adequate material for a cytological diagnosis. The patient underwent thyroidectomy, and histological examination disclosed a tumour in the left thyroid lobe, with the same pathological and immunohistochemical features as the previously excised caecal lesion (fig 2). Nonetheless, it was negative for Congo red, S-100 protein, and thyroglobulin stain; again, cdx-2 staining was positive, further confirming the caecal origin of this tumour (fig 3). Twenty one months after thyroidectomy, the patient died as a result of multiple organ failure.

To the best of our knowledge, this is the first case of a rare caecal NEC with metastasis to the thyroid to be reported. The differential diagnosis included several primary neoplasms. MTC is characterised by positive



**Figure 2** The caecal neuroendocrine tumour (right side of the figure) metastatic to the thyroid gland (on the left side) grew in a predominantly solid and trabecular architectural pattern, surrounded by a fibrovascular stroma. Haematoxylin and eosin stain; original magnification,  $\times 10$ .



**Figure 3** The thyroid metastasis was composed of spindle shaped cells with poorly defined cell borders, exhibiting scanty eosinophilic cytoplasm, salt and pepper nuclei, and inconspicuous nucleoli. The tumour cells showed intense nuclear staining for cdx-2; in contrast, thyroid epithelium was negative for this marker. Cdx-2 staining; original magnification,  $\times 40$ .

immunostaining for calcitonin<sup>4</sup>; nonetheless, calcitonin can also be produced ectopically.<sup>5</sup> In our patient, weak positivity for calcitonin was found at immunohistochemical examination; however, staining for thyroid transcription factor 1, a marker of thyroid or lung origin,<sup>6</sup> was negative, whereas cdx-2, a transcription factor involved in the proliferation and differentiation of intestinal epithelial cells encoded by a homeobox gene,<sup>7</sup> was positive, excluding MTC. Paraganglioma was ruled out by both the intense reactivity of neoplastic cells for cytokeratins, and the absence of sustentacular cells, as shown by negativity for S-100 protein.<sup>8</sup> Insular carcinoma could be excluded by the absence of a microfollicular pattern, the negative immunoreaction against thyroglobulin, and the positive immunostaining for neuroendocrine markers. Finally, a few cases of primary small cell carcinoma of the thyroid have been described,<sup>9</sup> which share identical pathological and immunohistochemical features with primary lung small cell carcinoma. Some of them are positive for calcitonin, and are therefore regarded as small cell variants of MTC. In our patient, small cell carcinoma was ruled out firstly because of patient history and also by positive immunostaining for cdx-2.

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## References

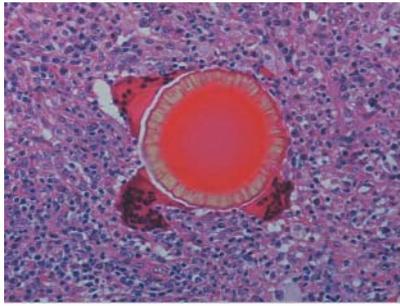
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## Liesegang rings in inflammatory breast lesions

We present two examples of Liesegang rings occurring in association with duct ectasia. Liesegang rings are a rare phenomenon usually found in association with cystic or inflammatory lesions, and may be mistaken for parasites.

The first patient, a 52 year old woman, had a radiological code 4 mass lesion on screening mammography. Needle core biopsy (NCB) showed breast tissue infiltrated by sheets of single cells, with abundant foamy cytoplasm and slightly eccentric nuclei. Cytological atypia was minimal and there was no significant mitotic activity. The cells were admixed with lymphocytes, plasma cells, and neutrophil polymorphs. Immunohistochemical studies showed that the lesional cells were strongly CD68 positive and cytokeratin negative, confirming the haematoxylin and eosin impression of an inflammatory process, and excluding histiocytoid carcinoma. The aetiology of the inflammatory process was not apparent on NCB and, in view of the radiological suspicion of malignancy, the patient proceeded to excisional biopsy. This revealed a 1 cm slightly irregular lesion with a white cut surface and yellow foci centrally, bordered by fatty breast tissue. Microscopically, the lesion was composed of an irregular dense aggregate of histiocytes, lymphocytes, plasma cells, and neutrophil polymorphs, as seen on NCB. Within the aggregate of inflammatory cells, foreign body type giant cells were identified, some of which were associated with round acellular structures. These structures typically comprised a double layered outer wall containing evenly spaced radial cross striations, surrounding dense amorphous non-refractile orangophilic material, interpreted as Liesegang rings (fig 1). There was evidence of fat necrosis and florid duct ectasia in the immediate vicinity. The overall histological appearances were thought to represent a predominantly histiocytic inflammatory process incorporating Liesegang rings, secondary to a ruptured ectatic duct. There was no evidence of malignancy.

The second patient, a 54 year old woman, had a radiological code 5 mass lesion in the upper inner quadrant of her right breast on



**Figure 1** Liesegang ring characterised by a double layered outer wall containing evenly spaced radial cross striations, surrounding dense amorphous non-refractile orangophilic material. The ring has developed within an area of chronic inflammation and is associated with a foreign body giant cell reaction.

screening mammography. After a needle core biopsy diagnosis of invasive ductal carcinoma with associated ductal carcinoma in situ, she underwent therapeutic wire guided breast wide local excision and sentinel lymph node biopsy. The breast specimen showed a 15 mm, grade 3, invasive ductal carcinoma, with extensive high grade ductal carcinoma in situ. Three sentinel lymph nodes were negative for metastatic carcinoma. The tissue lateral to the tumour showed features of duct ectasia. Liesegang rings were present in the lumen of one of the ectatic ducts and in the adjacent tissue with an associated foreign body type giant cell reaction.

Liesegang rings are laminated spherical ring-like structures that develop usually in relation to cystic or inflammatory lesions. The rings are typically composed of a mixture of calcium, iron, silicone, and sulfur and form by periodic precipitation from a supersaturated colloidal solution.<sup>1</sup> Liesegang rings are rare and have been described primarily in the setting of renal cysts,<sup>2</sup> but have also been observed occasionally in association with breast cysts, endometriotic lesions, and cysts at other sites.<sup>3-5</sup> In the above two cases, the Liesegang rings were related to duct ectasia and in the first case were an integral part of the mammographic lesion. Liesegang rings may be mistaken for psammoma bodies or parasites. Liesegang rings lack the internal organs of true parasites and have a characteristic histological configuration, as described above. Accurate identification of Liesegang rings supports the diagnosis of a cystic or inflammatory process, and decreases the possibility of erroneous misdiagnosis as another type of pathological process.

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Full consent was given for the publication of these cases.

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## Congenital bronchogenic cyst in the gastric mucosa

We read with interest the letter by Rubio *et al*, "Congenital bronchogenic cyst in the gastric mucosa" in the March 2005 issue.<sup>1</sup> In their report, the cyst they discovered contained pseudostratified ciliated epithelium with a lymphatic follicle. No cartilage was noted and no respiratory seromucous glands were mentioned. Although all bronchogenic cysts must have ciliated epithelium (pseudostratified ciliated columnar or cuboidal epithelium), they must also have cartilage or bronchial mucous glands.<sup>2-5</sup>

Foregut cysts include bronchogenic, oesophageal, gastroenteric, and pericardial types. The most common location for these cysts is in the mediastinum; however, cutaneous, cervical, diaphragmatic, abdominal, retroperitoneal, and gastric locations have all been described. Although gastroenteric and pericardial cysts are straightforward to differentiate, the distinction between oesophageal and bronchogenic cysts can be difficult because of their similar histological features, as a result of their close embryological development. All bronchogenic cysts must have ciliated epithelium (pseudostratified ciliated columnar or cuboidal epithelium). They also must have cartilage or bronchial mucous glands. Oesophageal cysts can have ciliated or non-ciliated epithelium of columnar, squamous, or mixed types. This epithelium sits on two well developed layers of smooth muscle with no cartilage or respiratory glands. When a cyst is only lined by ciliated columnar epithelium with none of the above mentioned distinguishing features, a foregut cyst is the appropriate description.<sup>2-5</sup>

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## Expression of HIF-1 $\alpha$ in human tumours

In their recent letter,<sup>1</sup> van Diest and colleagues make a valid point that the expression

of molecular markers in the literature is often discordant because investigators do not use standard methodologies. The use of tissue microarrays or whole tissue sections is one example of this, and van Diest and colleagues correctly point out that the core redundancy in tissue microarrays necessary for an accurate reflection of hypoxia inducible factor  $\alpha$  (HIF-1 $\alpha$ ) expression must be determined in a prospective fashion. Nevertheless, our evaluation of HIF-1 $\alpha$  staining was carefully controlled; we stained all tissues with a single antibody, at the same time, and used positive internal cell line standards on each section.<sup>2</sup> The assumption that the analysis of HIF-1 $\alpha$  expression in whole sections is prognostically superior to tissue microarrays is unfounded at this time. Indeed, a report by Torhorst and colleagues suggests that the assessment of biomarker status in arrayed tissue cores may carry greater prognostic value than assessment in whole sections.<sup>3</sup>

The objective of our analysis was to demonstrate that vascular endothelial growth factor (VEGF) is upregulated independently of activated HIF-1 $\alpha$  in most human tumours. This may imply constitutive overexpression or, more likely, reactive upregulation in response to other factors in the tumour microenvironment. The validity of this observation is not affected by the choice of tissue microarrays or whole sections. Indeed, a report by Mizukami and colleagues suggests that certain human cancers may exploit an HIF-1 $\alpha$  independent mechanism to upregulate VEGF in response to hypoxia.<sup>4</sup>

In summary, we strongly support any move that would help to standardise the reporting of the expression of molecular markers in tissues. However, we stand by our observations that the upregulation of VEGF in human tumours is largely independent of HIF-1 $\alpha$  activation.

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## CORRECTION

Salashor S, Woodgett JR. The links between axin and carcinogenesis. *J Clin Pathol* 2005;**58**:225-36. The third sentence of the abstract should read: "Overexpression of mutant axin..." and in fig 5 parts A and D are  $\beta$  catenin and B and E are axin 1. The authors apologise for these mistakes.