MVST BOD & NST Part IB **Thurs. 9th & Fri. 10th March 2017**
Pathology Practical Class 25

Problem-Solving Exercise (Neoplasia): Answers

The Molecular Basis of Cancer: Colorectal Adenocarcinoma

Overall, the key points of interest of this practical are to investigate how mutations are interpreted, the differences between patterns of mutations to oncogenes and tumour suppressor genes (TSGs); the relationship between mutations and the stages in development of a tumour; and inherited predisposition caused by inheritance of a tumour suppressor mutation.

It is important to appreciate that there are many forms of 'mutation' in cancer, not only point mutation, but larger-scale changes such as extra copies of chromosomes, chromosome translocations, deletions (of part or whole chromosomes), amplifications and 'non-mutational' alterations such as epigenetic changes (methylation of the promoters of genes that can "silence" their expression), all of which occur in colorectal cancer but are less well studied. The final picture **(Figure 5)** is intended to draw attention to this.

The *K-RAS* gene

In the tumour cells there are often (but not always) two alleles, one mutant and one normal.

Q1 Why are two bases detected at this position in the cancer?

Q2 Is K-RAS a tumour suppressor gene or an oncogene, and how can you tell from the data?

K-RAS is an oncogene, mutated in about 40-50% colorectal adenocarcinomas (and 40 – 50% adenomas). One copy is mutated and there is usually (but not invariably) retention of a normal copy. The sequencing trace **(Figure 2)** shows that there are 2 sequences present at position 436 (codon 146) including an A in the green trace and a G in the black trace, representing the mutant and normal sequences respectively. Note that the superimposed A and G peaks are approximately half the height of the adjacent peaks, as they are present in a single copy each, whereas the adjacent sequences are present in 2 copies. Table 1 shows that mutations in the *K-RAS* gene occur at relatively few sites (codons 12, 13, 146, etc) and result in activation of the K-RAS protein (confirmed by biochemical studies - see next paragraph) and are termed activating mutations. So this is an oncogene rather than a tumour suppressor gene.

Q3 Why are these mutations only found at particular amino acids?

Specific mutations are required to activate oncogenes. In the case of the K-RAS protein, which binds GTP to form the K-RAS-GTP active conformation, the mutations act by inactivating its GTPase enzymic activity without interfering with its other functions. In fact, the mutations interfere with the potentiation of GTPase activity by GAPs (GTPase activating proteins). This allows K-RAS protein to stay constantly bound to GTP,

remaining in its active conformation, sending growth signals to the nucleus (normally the GTPase activity cleaves off a phosphate group changing it to K-RAS-GDP – the inactive state).

The *APC* gene

Q4 How frequent are APC mutations in these tumours? 35 tumours were screened; note some tumours have 2 mutations.

There are 25 colorectal cancers showing a total of 33 mutations in 35 tumours tested. Note that several tumours have two mutations, presumably in most cases on two different alleles (note that this is at odds with the usual story that one allele is truncated and one is lost altogether). This ratio is 25/35 = 71% of cancers showing *APC* mutations (1 or 2 per tumour). All *APC* mutations were found in the colorectal cancers, with no breast cancers showing *APC* mutations.

Q5 What do these mutations do to the protein encoded by the gene, in particular, what would be the consequence of the INDEL (insertion or deletion of one base pair) mutations?

Mutations in *APC* most often truncate the protein by introducing a STOP codon. This happens either by an amino acid codon being changed to a STOP codon (a 'nonsense' mutation), or by a frame shift (insertions or deletions of 1 or 2 bases also known as INDELs that produce a frame shift), which will result in a STOP codon soon after the frame shift because genes have many stop codons in the invalid reading frames (previously out-of-reading frame but now brought in-to-reading frame by the INDEL).

Q6 Compare the range and type of mutations found in APC with the range and type of mutations found in K-RAS. Is APC a tumour suppressor gene or an oncogene, and how can you tell from the data?

Table 1 shows the wide range of different *APC* mutations at many different positions along the gene (unlike those in *K-RAS*), these mutations are inactivating (nonsense or frame shift mutations producing STOP codons leading to truncation of the protein) and that many mutations are homozygous (i.e. there is only mutated *APC* present) or else the tumour has two different mutations which will usually be on different alleles of the gene (unlike those in *K-RAS*). *APC* is not an oncogene because there are no copies of the normal gene retained: both copies are altered and inactivated. (Please note that some tumours may show a combination of mutation of 1 allele and deletion/loss of the other allele and so the mutation appears to be homozygous).

Figure 3a shows a Western blot of APC proteins from colon cancer cells, and other cancer cells. On these blots, the further the band migrates (downwards) into the gel by electrophoresis, prior to blotting, the smaller the protein.

Q7 Why are the proteins different sizes; why do the colonic tumours (usually) have only one band (one size of protein); why are the pancreatic tumour bands all the same size? Do you think this is typical for a tumour suppressor gene?

The truncation mutations result in short proteins which then migrate faster through the gel on electrophoresis, giving the altered band positions in a Western blot. The truncation mutations can occur at many different points, hence the wide variations in protein sizes which show as different band positions in the Western blot. Only one band is (usually) seen, because one copy of the gene has been mutated, the other copy is commonly (and in all the cases shown in this blot) completely lost by deletion of a segment of the chromosome. The pancreatic and prostate tumours do not have *APC* mutations. This pattern of mutation, with almost all mutations truncating the protein and no cases with complete loss of the protein, is not typical for a tumour suppressor gene (it indicates that there may be residual function for the truncated protein). For example, when *p16/INK4* is mutated it is often completely deleted, and the pattern of mutation in *TP53* is different again.

Q8 Why are APC mutations more variable than K-RAS mutations?

K-RAS mutations have to activate the protein, which requires mutation to particular amino acids at particular positions (e.g. in the GTPase active site) in the protein. Tumour suppressor genes generally can be inactivated in many ways, and even for *APC*, which seems to require to be truncated, there are many ways to make a truncation with nonsense mutations or frame shift mutations at many different positions along the gene.

Q9 Why would the APC gene be named after the disease?

Because the gene was first identified and cloned using mapping of the FAP/APC disease locus as a crucial step to locate the position of the *APC* gene on chromosome 5 (there is a different gene already called *FAP* so this gene was called *APC*).

Q10 What would we expect to find if we sequenced the APC gene in germ-line DNA of a patient with FAP, and a colonic adenoma or cancer from that patient? If we sequenced germ-line DNA in a more typical sporadic colon cancer patient, how would the result be different?

FAP is a classic example of Knudsen's "Two Hit Hypothesis" for tumour suppressor genes like *Rb-1*, the gene for inherited susceptibility to Retinoblastoma. FAP patients inherit a mutated *APC* gene in every cell in the body so they have a very high probability of having colorectal epithelial cells that by losing or mutating their normal copy of *APC* end up with only mutant *APC* and are on the road to an adenoma.

So in the germ-line of a FAP patient we should find one mutant and one normal copy of the *APC* gene, while in an adenoma or cancer we would find only the mutant form. In a more typical sporadic colorectal cancer patient the germ-line DNA would show no mutations in *APC.* It is important to note that all patients with sporadic colorectal cancer have NOT inherited an *APC* mutation (only those with FAP inherit a germ-line mutation in one copy of *APC*).

Figure 3b shows a Western blot of APC protein in lymphocytes from FAP patients. Compare these with the tumours.

Q11 What are the bands, why are there two bands in the lymphocytes but only one band in the tumour?

Lymphocytes will have the same *APC* genes as the germ-line. So lymphocytes (or lymphoblastoid cell lines made from them) from a normal individual will make only normal APC protein, whereas lymphocytes from an FAP patient will be making *both* normal and mutant APC protein, in contrast to tumours, which have lost the normal *APC* gene.

Q12 Suppose someone is discovered to have Familial Adenomatous Polyposis, and there is concern that some of their close relatives may also be affected. How would you identify the affected relatives?

If someone is suspected of belonging to an FAP family they would usually be examined by colorectal endoscopy, which reveals hundreds of adenomatous polyps in the large intestine (usually in the late teens or twenties – see **Figure 4**). They might have a family history. To identify the affected relatives, testing of their germ-line DNA (extracted from blood) can be performed for the known mutation in their family, but for this you would need to know where the mutation is, by sequencing the *APC* gene in the known FAP case in the family, and this is technically difficult even today, though maybe not in future.

Q13. Tricky question. Suppose a family has a clear history of inherited predisposition to colorectal cancer. Are they likely to have FAP, i.e. an APC mutation, or what other explanation would you consider first?

Not all hereditary predisposition to colorectal cancer is due to inherited APC mutations: in fact far commoner is Lynch Syndrome (old name HNPCC) which is inheritance of a mismatch repair deficiency. These patients do not show polyposis (the presence of very large numbers of polyps in the intestines) they may show only a few polyps or no polyps prior to colorectal cancer formation, so endoscopy wouldn't pick up affected relatives.

The PI3KCA gene

Q14 Compare the range and type of mutations found in PIK3CA with the range and type of mutations found in K-RAS and APC. Is PIK3CA a tumour suppressor gene or an oncogene, and how can you tell from the data?

The range of mutations in *PI3KCA* is narrow (affecting positions 542, 545, 901, 907 and 1047) and these are missense mutations (rather than nonsense or frame shift mutations), indicative of the pattern of mutations seen affecting an oncogene (similar to those in *K-RAS,* but with a slightly bigger range of sites affected). Thus, *PI3KCA* is an oncogene. The mutations are thought to activate the gene. In contrast, in tumour suppressor genes the mutations are usually nonsense or frame shift mutations, occurring at a much wider variety of positions along the gene, and these mutations lead to inactivation of the gene (along with small or large scale deletions or promoter methylation changes).

Larger-scale DNA alterations : Figure 5

Figure 5 shows the chromosomes of a cultured cell line derived from a colonic cancer (obtained in Dr Edwards' lab). Each chromosome is marked with a different fluorescence colour.

Q15 How many pieces of chromosome 5 are there (coloured brown).

There are at least six fragments of chromosome 5 coloured brown (in the chromosome 5 box), with some fragments of chromosome 20 (dark blue) joined to some of the chromosome 5 fragments (in the chromosome 5 box); and there is a complex translocation with parts of both chromosome 11 and chromosome 5 along with part of another chromosome 3 (in the chromosome 11 box). The four chromosome translocations (in the chromosome 5 box) made up of bits of chromosomes 5 and 20, appear to be two copies each of a reciprocal translocation between 5 and 20 (hence 4 copies).

Q16 Notice that several chromosomes are present in four copies and several of the abnormalities are present in two copies. Can you think of a way this could have happened?

Cells sometimes duplicate their entire genome, and this is well documented in carcinomas - it seems likely that such a duplication event has occurred during the evolution of this cancer cell karyotype (although there is often some subsequent loss of chromosomes and gain of other chromosomes or chromosome translocations, which accounts for the less than perfect collection of 4 copies of all chromosomes).