

# Analysis of the WT1-VEGF molecular pathway in Acute Myeloid Leukaemia



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## Introduction:

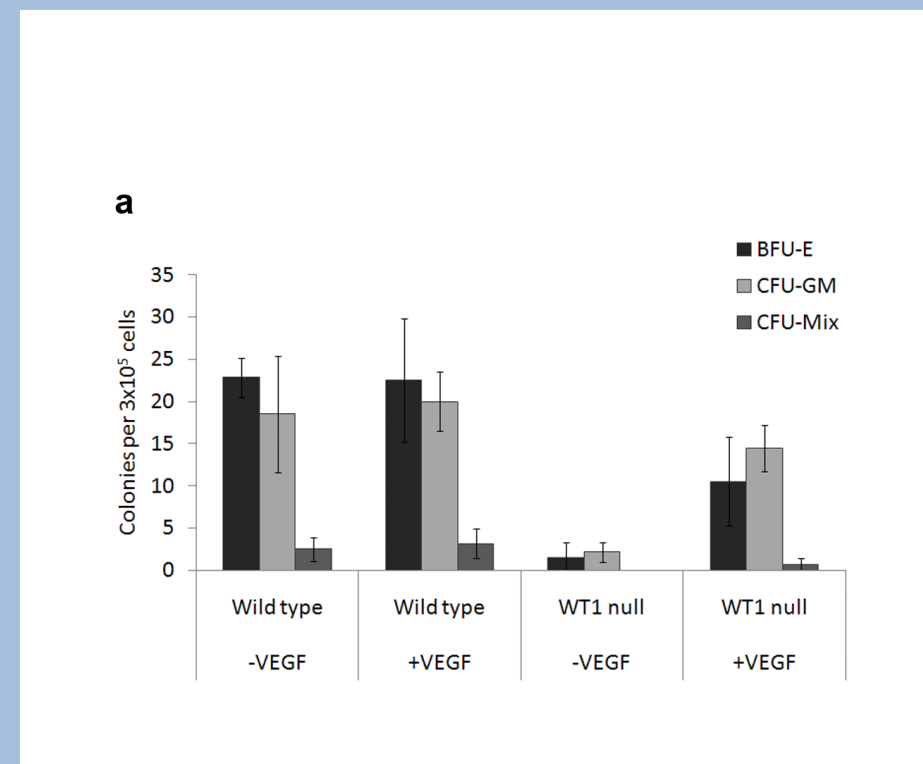
WT1 regulates VEGF in blood development [Figure 1].

WT1 mutations and altered VEGF predict poor outcome in AML [Reference 1 & 2].

In kidney cells, WT1 is part of a molecular pathway including Srpk1 [Reference 3].

Figure 1.

Absence of WT1 (WT1 null) severely reduces blood development from mouse embryonic stem (ES) cells. Graph shows the number of blood progenitor cell colonies is ~20% that derived from wild type ES cells and that addition of VEGF protein to the cultures can partly restore blood development in WT1 null ES cells. This result highlights the importance of the WT1-VEGF pathway in blood development. [Thomas Cunningham, PhD Thesis, Newcastle University]



## Aims:

- To find relative levels of VEGF and Srpk1 in AML samples
- To assess if changes in levels correlated with WT1 mutations
- To analyse AML samples for evidence of novel WT1 mutations

## Results and Methods:

Testing oligonucleotide primers for RT-PCR using human podocyte cDNA and AML patient cDNA alongside a water blank. VEGF 4.1/8.2, VEGFxxx and SRPK1 oligos were selected for the remaining analyses.

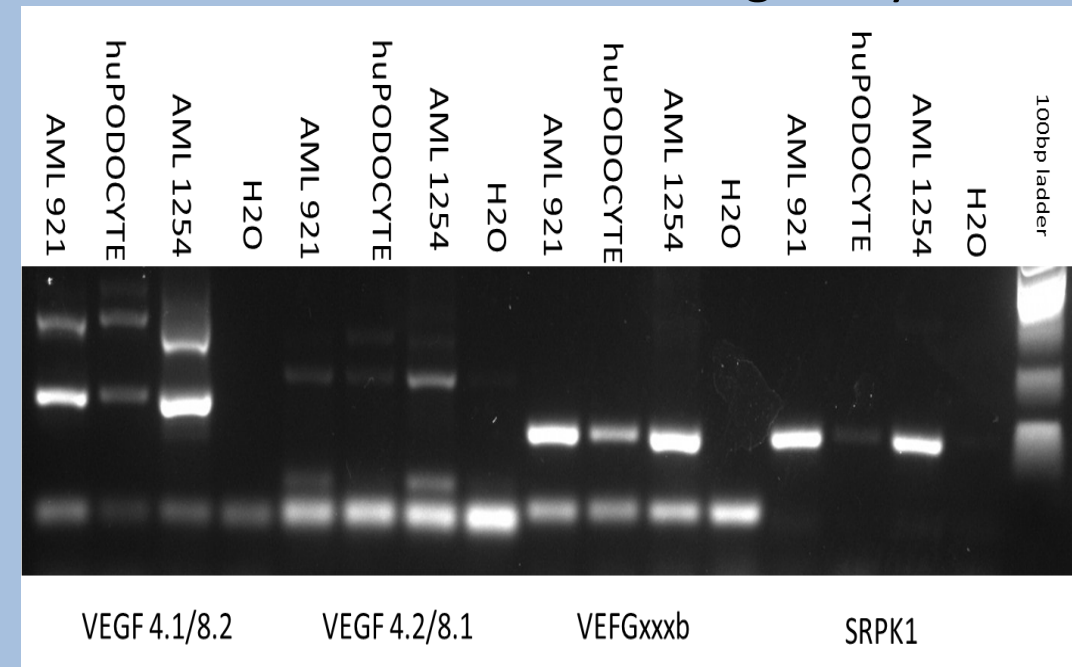
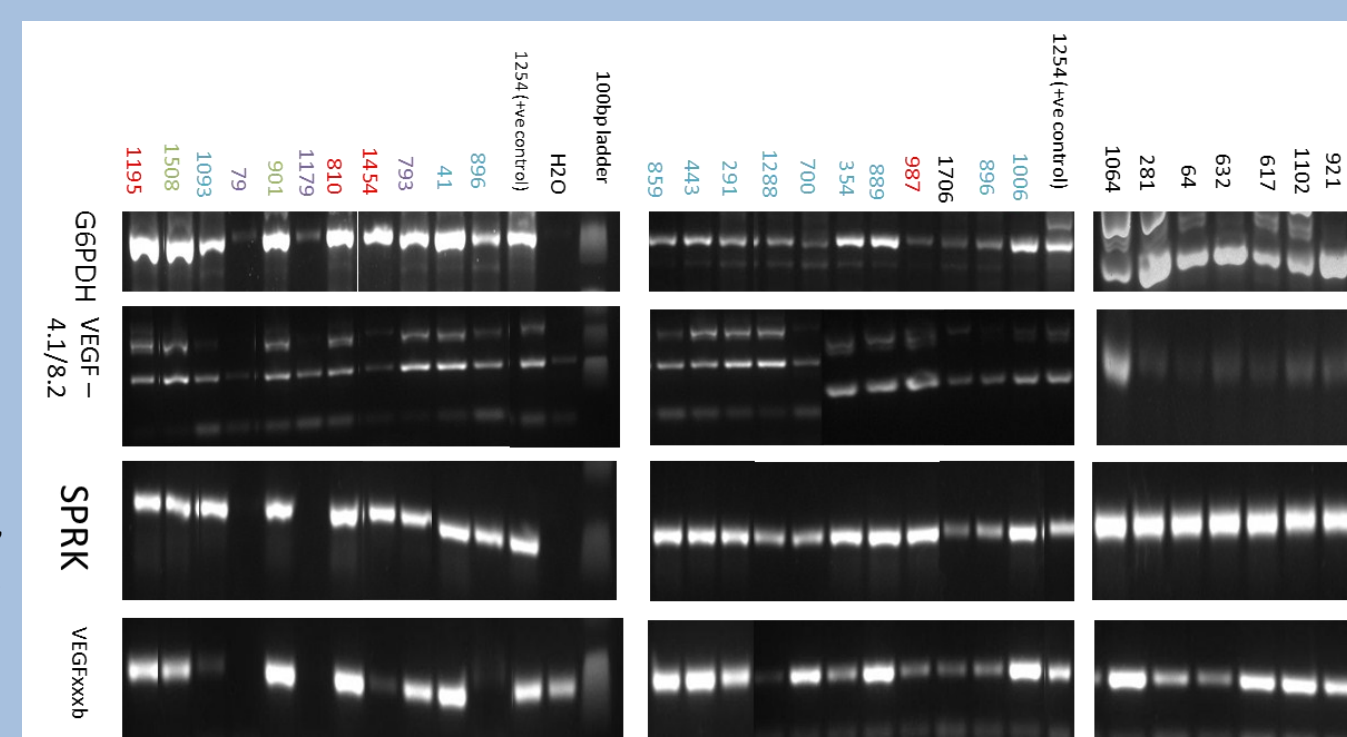


Figure 2.

This figure shows the results of an initial PCR reaction to determine if the oligos worked with the AML samples by comparing with a human podocyte cDNA sample known to express the genes of interest. The products were run on a 2% agarose gel and visualised with Gel red and UV light. SRPK1 and VEGFxxx bands are ~100bp whilst VEGF 4/8 primers produce several bands between 100bp and 500bp due to alternative splicing.

Figure 3.

Composition of gels to show relative levels of VEGF 4/8, VEGFxxx and SRPK1 in AML samples. G6PDH was used to determine the amount of cDNA in samples to allow interpretation of the expression of the genes of interest. In the majority of cases, the intensity of the bands are proportional to the loading control (G6PDH), however, eg, 1454 shows a lower expression of VEGFxxx than expected, 896 looks to correlate to this pattern but not as strongly.



1234 = Inv(16) mutation, 1234 = FLT3+ve mutation, 1234 = 16;16 mutation, 1234 = M2 mutation, 1234 = point mutation, 1234 = unlisted mutation

Samples were also analysed to identify any changes in the WT1 sequence, oligos were used to look at the N and C terminals as well as looking for exon 9 splicing. The results are shown in the figure below.

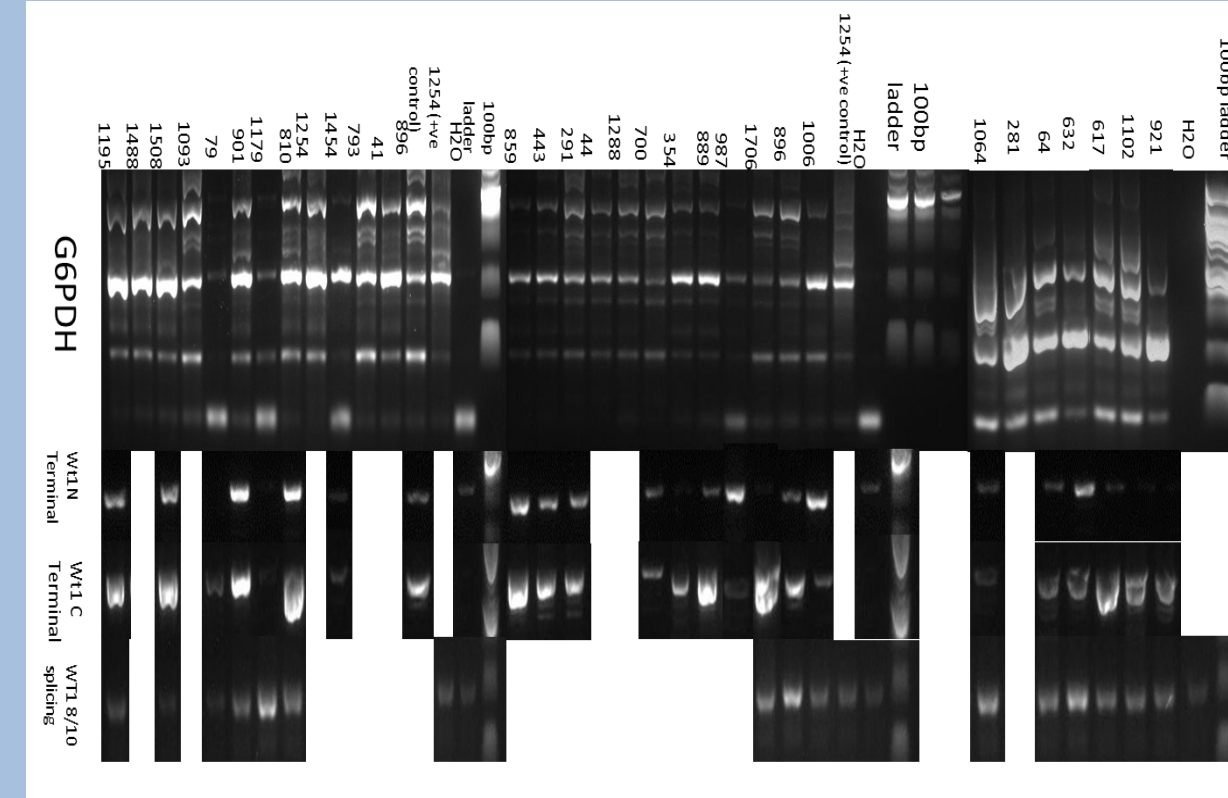


Figure 4.

The samples were checked for baseline cDNA levels using the same methods as previously. They were then PCR'd with oligos for the N and C terminals as well as for exon 9 skipping. Once the samples had been run they were analysed for candidate samples to be sent for sequencing by GATC technologies. Samples 1254 and 896 were chosen as positive and negative controls for exon 9 skipping. Samples 810, 987 and 354 were chosen as initial samples for C terminal sequencing. White spaces indicate where tests were not run due to lack of sample material.

In order for the samples to be sequenced they needed to be purified and made to the correct concentrations. Purification of the PCR fragments was done using a QUIAQUICK kit, the initial samples were then run against a 1kb ladder with known concentration in its bands, the amount of cDNA in each sample could then be roughly estimated by comparing to the ladder. The samples also needed to include a small amount of primer, as this would allow the sequence to be read from a certain point, samples were diluted down to 5picomoles and mixed with the purified DNA samples and then sent off. Once the sequences had been analysed they were visualised to .abi files and could be viewed in the GATC viewer to compare with the wild type sequence to see if any changes had occurred. To check the wild type samples they were screened for KTS. (figure 5.)

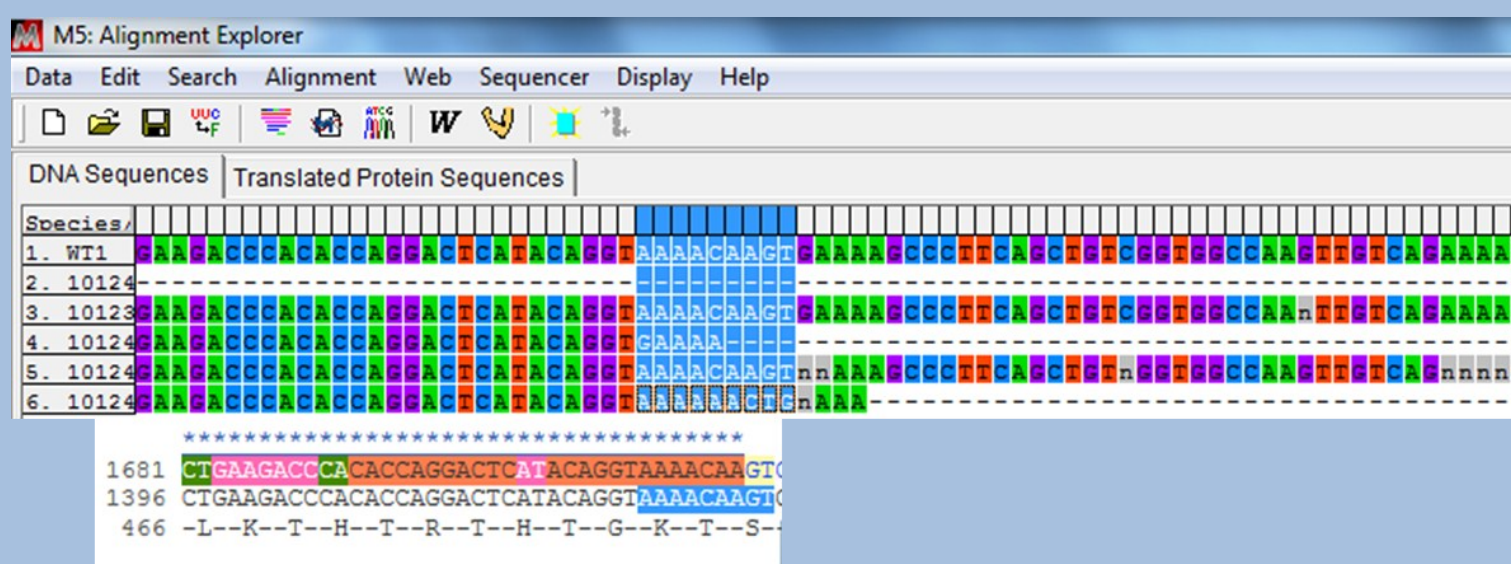
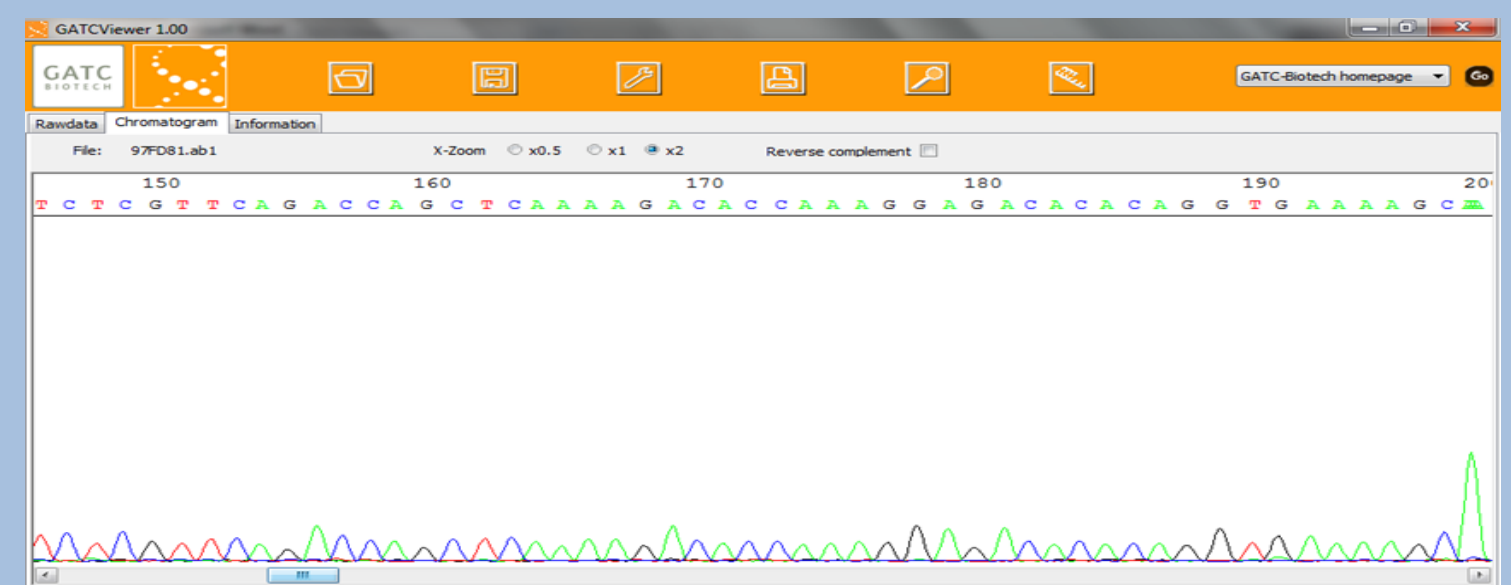


Figure 5.

Samples were run through MEGA5 to visualise the sequence and to make it easier to locate the wild-type KTS coding sequence. The sequence is shown below the MEGA5 window with the KTS sequence being highlighted in blue. Direct comparison allows us to prove that the samples were wild-type as the KTS sequence was present. The GATC visualization shows the sequence and levels of bases from the sample.



Following KTS checking, the mutant WT1 samples were checked to ensure they included exon 9 splicing. This was done using the same method previously mentioned. The results showed that splicing did occur in the samples, the sequence to look for was found by looking up the wild-type WT1 sequence and manually combining the sequences from exon 8 and exon 10. The spliced sequence was determined by using BLAST data available online, by determining the exons (coding sequences) from the introns (non-coding regions) it was possible to find where the sequences for exons 8 and 10 would join together. The samples did show the spliced sequence and so we are able to confirm the presence of exon 9 splicing in some AMLs. (figure 6.)

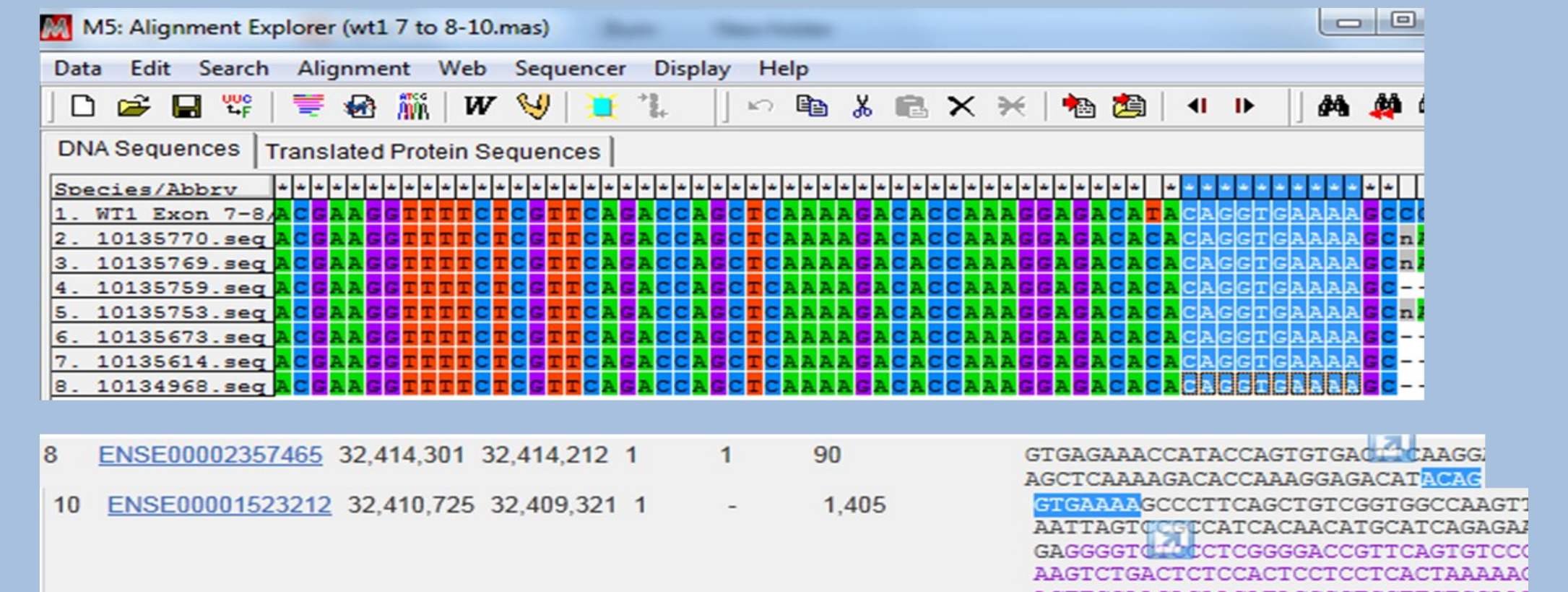
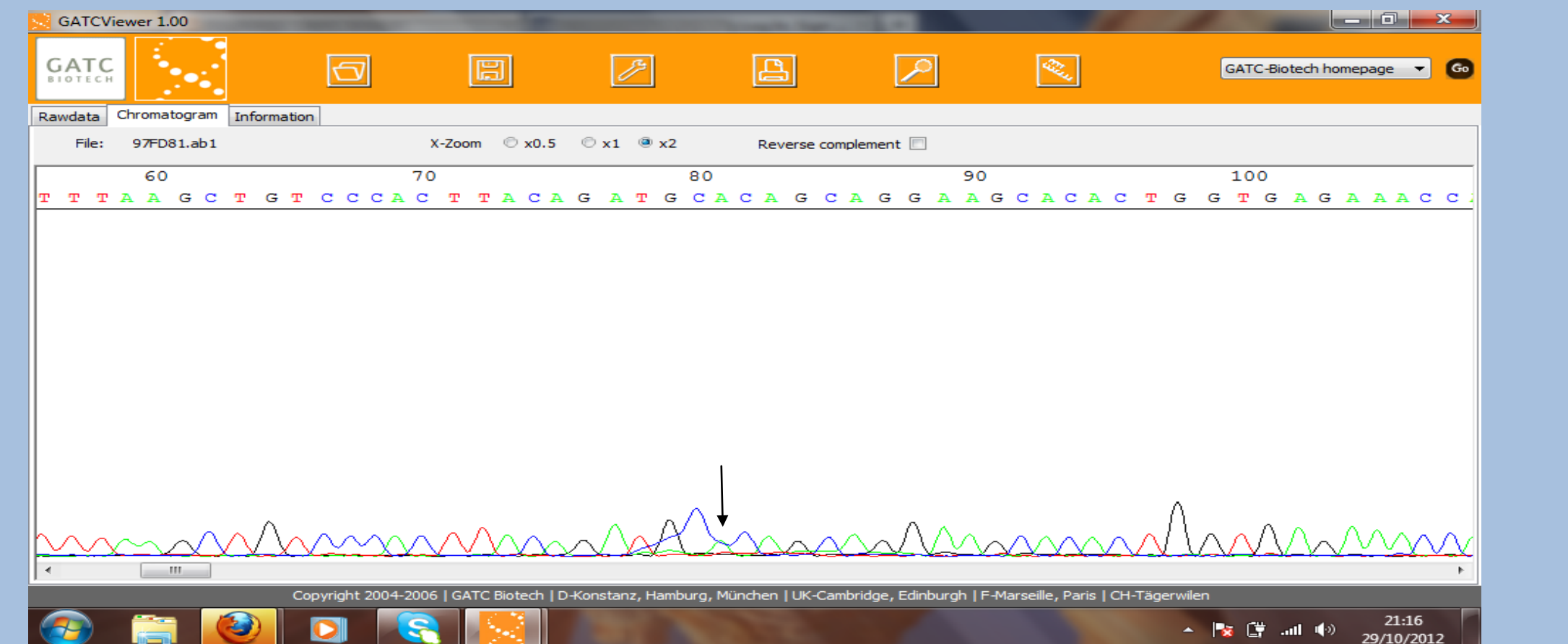


Figure 6. MEGA5 analysis of spliced samples to check that exon 9 had been spliced from the WT1 sequence. The highlighted regions show the spliced sequence that confirms the exclusion of exon 9 from the WT1 coding sequence.

After checking that exon 9 had been spliced each sample was visualised through the GATC viewer and compared to the expected sequence to look for any mutation, only one sample (1706) showed variation, the screenshot is shown below and suggests a base change on one of the replicating DNA strands. (figure 7.)



The sample suggest a single amino acid change at position 82 from an C to an A. This would convert from a CAC to CAA—changing the amino acid from a histidine to a glycine. This change could have a possible role in the worsened prognosis of WT1 exon 9 spliced AMLs. However this would need further investigation—capillary gel electrophoresis could be used to further single out the fragment to allow for re-sequencing to confirm a change which could be moved forward with.

## Conclusions:

One of the samples - 1706 shows a possible variation from the wild type sequence, a C to A conversion at position 82. 1706 shows normal levels of srpk1 and vegfxxx. Although other samples show more variation in levels of Sprk1 and Vegfxxx they do not show any variation from the wild type sequence. This could be further explored and may possibly have an affect on the prognosis of WT1 exon 9 spliced AMLs—if shown to have a regulatory effect this could be biochemically manipulated to return normal function to the pathway and increase chances of a better prognosis.

## REFERENCES

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- Virappane, P., et al. Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: the United Kingdom Medical Research Council Adult Leukaemia Working Party. *J Clin Oncol* 26, 5429-5435 (2008).
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