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SHORT COMMUNICATION

A novel optical biosensor format for the detection of clinically relevant TP53 mutations

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Abstract

The TP53 gene has been the subject of intense research since the realisation that inactivation of this gene is common to most cancer types. Numerous publications have linked TP53 mutations in general or at specific locations to patient prognosis and therapy response. The findings of many studies using general approaches such as immunohistochemistry or sequencing are contradictory. However the detection of specific mutations, especially those occurring in the structurally important L2 and L3 zinc binding domains, which are the most common sites of TP53 mutations, have been linked to patient prognosis and more strongly to radiotherapy and chemotherapy resistance in several major cancers. In this study, the TI-SPR-1 surface plasmon resonance system and Texas Instruments Spreeta™ chips were used to develop a DNA biosensor based on thiolated probes complementary to these domains. The sensors were able to detect these mutations in both oligonucleotides and PCR products with normal and mutant TP53 DNA, but the difference in hybridisation signal was small. Preliminary experiments to enhance the signal using *Escherichia coli* mismatch repair proteins, MutS and single strand binding protein were carried out. It was found that MutS was unable to bind to mismatch oligonucleotides, but single strand binding protein was able to bind to single stranded probes which had not hybridised to the target, resulting in a 3-fold increase in the sensitivity of the biosensor. While

further work needs to be carried out to optimise the system, these preliminary experiments indicate that the TI-SPR-1 can be used for the detection of clinically relevant mutations in the TP53 gene and that the sensitivity can be increased significantly using single strand binding protein. This system has a number of advantages over current mutation detection technologies, including lower cost, ease of sensor preparation and measurement procedures, technical simplicity and increased speed due to the lack of need for gel electrophoresis.

Keywords: TP53 mutation, p53 mutation, DNA biosensor, surface plasmon resonance, mutation detection, optical biosensor

1. Introduction

The human genome mapping project has facilitated and driven the development of many new technologies for molecular diagnostic applications, including immunoassays, protein assays, genetic analysis and gene expression studies (Gander *et al.*, 2003). These tools have the potential to be used and have in some cases already been used widely in medical diagnostics, particularly in cancer diagnostics. It is highly desirable to be able to determine the presence of cancer in asymptomatic patients, how aggressive the tumour is, whether there are metastases, the preferred treatment, effectiveness of surgery, the response to chemotherapy and recurrence of tumours (Gander *et al.*, 2003). A major area where genetic tests could be applied is in prognosis and therapy selection and to that end a large amount of research has been carried out to link genes and genetic mutations to cancer pathogenesis and response to treatment. The human TP53 gene is a prime example of this, as extensive research has revealed that this gene is the most frequently mutated gene in human cancers and it is thought to be inactivated or function abnormally in the majority of tumours. This can be observed by either increased concentration of p53 protein, complete absence of p53 protein or the presence of genetic mutations or mutated forms of p53 protein. Not only does mutation disable the normal TP53 functions, including cell cycle arrest and induction of apoptosis in response to DNA damage and inhibition of angiogenesis, but some mutations

can also provide tumours with growth advantages and resistance to chemotherapy and ionising radiation. Although the prevalence of different TP53 mutations varies between cancer types and tumour subclasses, the most common mutations occur at “hotspot” codons on the gene. These mutations can be detected either by analysing the p53 protein structure, the level of p53 protein expression or all or part of the TP53 genetic sequence. The advantage of full-length sequencing is that no mutations are missed, but this procedure is time consuming. In addition, the quicker sequencing methods and other mutation detection technologies are not always accurate, are very technically demanding or have to be repeated using different conditions to obtain maximum sensitivity. Short oligonucleotide probes can be used in a number of ways to detect particular mutations with a high sensitivity or can be combined with other probe sequences to rapidly detect mutations at several sites at once. This technology is ideally suited to the detection of gene mutations which can occur at a number of particular codons, such as TP53 mutations.

A number of studies have found that mutations in specific structural domains of the TP53 gene are linked to patient prognosis and response to specific therapies and where there is sufficient evidence that this is the case a clinician could use a diagnostic test to select the most appropriate treatment for individual patients (Soussi & Béroud, 2001). The biological justification for this, areas where it might be applied and the mutation detection technologies currently available for this application, have recently been reviewed (Jiang *et al*., 2004a).

The aim of this study was to investigate the potential of surface plasmon resonance as a means of detecting point mutations in the TP53 gene with a view to the development of sensors to detect mutations within the structurally important and clinically significant regions where TP53 mutations occur. Such a sensor could have value in both assessing prognosis and therapy selection for cancer patients. We sought to show that the detection of single base mutations in the TP53 gene using a surface plasmon resonance DNA biosensor based on the Texas Instruments Spreeta^{TM} system, could be

enhanced by the application of the mismatch binding protein MutS and single stand binding protein.

2. Materials & Methods

The TI-SPR-1 (Texas Instruments, USA) optical biosensor system was used with Spreeta^{TM} sensor chips coated with thiol-immobilised DNA probes (Sigma-Genosys, Cambridge, UK) in a flow cell format (see Figure 1) and controlled by Spreeta^{TM} software. The 28-mer DNA probes corresponded to the structurally important L2 zinc binding domain of the TP53 gene (sequence 3'--HS-$(CH_2)_6$-CTCCAACACTCCG**C**GACGGGGGTGGTAC--5' and were used to detect hybridisation of oligonucleotide probes (Sigma, Milan, Italy) identical to both the normal TP53 sequence (5'--GAGGTTGTGAGGC**G**CTGCCCCCACCATG--3') and the most common mutation in this region, a (G → A SNP at codon 175) **(**5'--GAGGTTGTGAGGC**A**CTGCCCCCACCATG--3'). Sensor chips were prepared by cleaning with ethanol followed by 10 minutes incubation in a solution containing ethanol, 30% ammonia, and MilliQ water in a 1:1:5 ratio. After drying the chips were inserted into the TI-SPR-1 and the flow cell connected. Thiloated probe (1µM) in potassium phosphate buffer (KH_2PO_4, pH 3.4) was then passed over the sensor surface for 2 hours at 5µl / minute**,** followed by 1 hour exposure to 6-mercapto-1-hexanol in darkness at the same flow rate. The chips were stored in flowing MilliQ water (2µl/minute) overnight and exposed to hybridisation buffer for at least 30 minutes before use, in order to obtain a stable baseline. The high reproducibility of chips produced by this method has been demonstrated in previuosly reported work from our group (Jiang *et al*., 2004).

Hybridisation was carried out by establishing a stable baseline in hybridisation buffer and then flowing oligonucleotides (1µM) in hybridisation buffer over the sensor surface for 5 minutes. This was followed by 3 minutes in hybridisation buffer to compensate for the bulk effect of the probe solution and to wash away any unbound material from the sensor surface. Finally the sensor was regenerated by 2 minutes exposure to 25mM NaOH and returned to

immersion in hybridisation buffer in preparation for the next measurement. A flow rate of 10µl/minute was used throughout. The hybridisation signal was taken as the difference between the refractive index at the baseline and that at the end of the 3 minute washing step. The mismatch binding protein MutS and single strand binding protein (SSB) (Amersham Biosciences, Milan, Italy) were applied after hybridisation to enhance the sensitivity to mutations. The proteins were made up in similar solutions to those in other studies; MutS (0.22µM) in MutS binding buffer ((50mM TRIS-HCl, 100mM KCl, 1mM (EDTA), 1mM dithiotreitol (DTT), 5mM $MgCl_2$, pH 7.4) and SSB (0.29µM) in SSB binding buffer (50mM TRIS-HCl, 100mM KCl, 1mM EDTA, 1mM DTT, 5mM $MgCl_2$, 0.005% TWEEN 20, pH 7.4). Measurements were carried out by exposing the sensor to the protein for 15 minutes at a flow rate of 2µl/minute following hybridisation The sensor was then washed in hybridisation buffer for 3 minutes at a flow rate of 2µl/minute and 9 minutes at 10µl/minute, with the signal being recorded at the end of the 9 minute washing step. A negative control experiment was carried out by applying SSB prior to the MutS to inhibit MutS binding to single stranded DNA and DNA chain ends. In addition, 0.29µM bovine serum albumin (BSA) (Amersham Biosciences, Milan, Italy) in MutS binding buffer was used as a negative control for all protein experiments because it is a similar size to MutS, can adsorb strongly to bare gold surfaces and exhibits no specific binding to any of the reagents used in this sensor system. Figure 1 shows a diagram describing how these experiments can be configured as a detection system.

3. Results

Our previous work has shown that the TI-SPR-1 is sensitive enough to detect differences in hybridisation affinity caused by single nucleotide polymorphisms (SNPs) and in particular those relating to TP53 mutations (Jiang *et al*., 2004b). In this study, the mutant DNA sequence containing a single base mutation produced a refractive index change (RIC) discernible from that of the normal DNA sequence on any given Spreeta^{TM} chip. However, this difference was relatively small and slight variations between chips may necessitate calibration of each individual chip for reliable routine use.

In this study MutS and SSB were applied in an attempt to enhance the sensitivity so that clear cut-off points for RICs indicating the presence of a TP53 mutation in the L2 zinc binding domain could be established. If applied after hybridisation, MutS should give a higher RIC if a mutation is present than if no mutation is present, since mutations create mismatched DNA sequences. SSB should also give a higher RIC when a mutation is present, because mutant DNA has a lower affinity for the immobilised DNA probes and less hybridisation occurs, so there are more free probes on the sensor for the protein to bind to. Application of SSB prior to MutS should not affect the ability of MutS to bind to mismatches, but would prevent non-specific binding of MutS to DNA probes and chain ends.

The results of these experiments (see Figure 2) show that when MutS was applied directly after hybridisation there was no clear difference in binding signal between mutant and normal DNA sequences. Furthermore there was no MutS binding following the application of SSB. This indicates that MutS was binding to either DNA chain ends or immobilised DNA probes, possibly because the high density of immobilised probes on the sensor surface prevented the relatively large MutS protein binding to mismatches. However, there was a clear difference in SSB binding between mutant and normal DNA and this difference was three times greater than the difference resulting from the hybridisation signal alone.

The stability, optimum storage conditions and reproducibility of the sensor were investigated in detail and it was found that it produced a stable baseline through at least 50 regeneration cycles, high reproducibility of hybridisation signal and could be stored for at least 1 night in flowing MilliQ water (2µl/minute) with no significant loss in sensitivity (data not shown).

4. Discussion

The inability to detect mismatches using MutS protein in these experiments indicates that when MutS was used alone it was binding either to the ends of duplexes formed by hybridisation or to single stranded free probe, as was

observed in previous studies (Gotoh *et al.*, 1997, Babic *et al.*, 1996). The second explanation would seem the more likely since the average signal obtained with MutS applied to complementary oligonucleotide was higher than when a mismatched sequence was used. This is supported by the very low signal obtained with BSA, which showed that all the proteins were interacting specifically with the immobilised probes and/or hybridised oligonucleotides, as BSA adsorbs well to bare gold surfaces and indeed has been used as the basis of an immobilisation procedure for Spreeta™ sensors (Kukansis *et al.*, 1999). We suggest that the relatively large (91kDa) MutS protein was unable to reach hybridised DNA at the sensor surface due to the high density of immobilised probes and so was unable to bind specifically to mismatches in this case. MutY, a protein of the same family as MutS, has been used in a solid phase assay for the detection of TP53 mutations (Lu & Hsu, 1992) and it is possible that MutS could also be used in this application if the conditions required for binding can be optimised.

The difference in signal between SSB binding with complementary and mutant oligonucleotides was much greater than expected. This can be explained by taking into consideration the fact that the hybridisation conditions used were optimised to produce the maximum difference in oligonucleotide binding and this difference would be amplified by the binding of SSB since this protein is larger than the oligonucleotide strands and binds close to the sensor surface. The SSB experimental data reported here is based on preliminary tests and further investigation is required to establish the optimum hybridisation conditions and SSB exposure parameters and to determine the sensitivity with different concentrations of oligonucleotides and PCR products. While the method is a little more complex and less reproducible than monitoring hybridisation directly, it is still much simpler and quicker than many of the mutation detection methods described by Nollau & Wagener (1997) and the remaining difficulties could be resolved easily by the use of an automated liquid handling system.

5. Conclusions

While the prognostic and therapeutic indications of TP53 mutations are still subject to debate, in some cancer types clear patterns are emerging where patient response to therapies which cause cancer cell apoptosis through the TP53 pathway is considerably reduced when a TP53 gene mutation is present. Current cancer research on the TP53 gene and P53 protein reveal significant associations between mutations and patient outcomes when mutations are present in the structurally important L2 and L3 zinc binding domains in breast, colon, head and neck and lung cancer, wheras other types of mutation are not associated with patient outcome at all or are specific to a certain cancer type. The likelihood is that further studies into the significance of TP53 mutations in other cancer types will reach similar conclusions. Therefore at present a detection system for mutations in these specific regions seems the best approach to develop a test with the widest possible area of application.

In this study a region of the L2 zinc binding domain was chosen as a target region as it was of appropriate length for the analysis and is the site of the second most common mutation in the TP53 gene, a G-A substitution at codon 175. The demonstration of a successful mutation detection system using probes corresponding to this mutation leads to the assumption that mutations of different types throughout the L2 domain could also be detected, and this will be verified in future work.

Overall, this study suggests that an optimised system based on the application of SSB after hybridisation could be utilised in the detection of these clinically relevant mutations. Such an approach has the advantage of needing inexpensive equipment that would be relatively straightforward to automate and could provide rapid results, with high sensitivity.

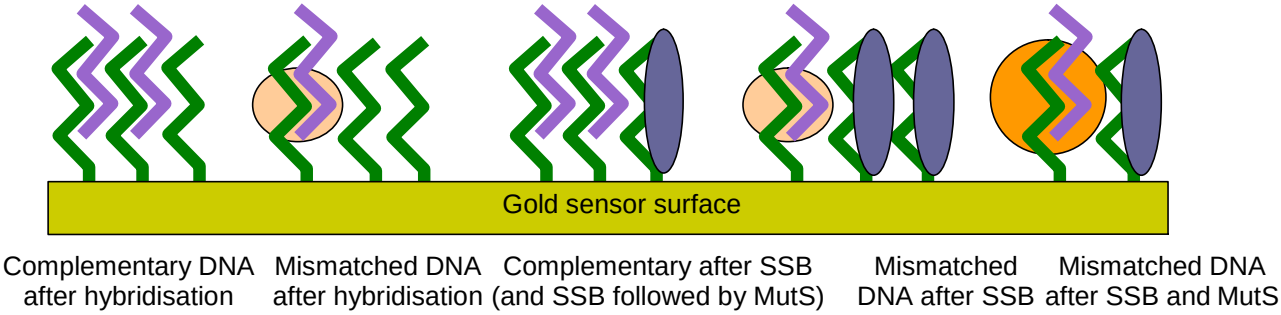

Figure 1: The Detection System. The diagram illustrates the sensor surface following each of the different types of assay and shows that the material bound at the sensor surface is likely to be different in each case. Lines connected to the sensors surface represent immobilised DNA probes, free lines represent target DNA, small circles indicate unstable binding regions, ovals represent SSB protein and large circles represent MutS protein. Complementary DNA followed by SSB and SSB followed by MutS would produce the same result if MutS bound only to mismatches, as no mismatches are present.

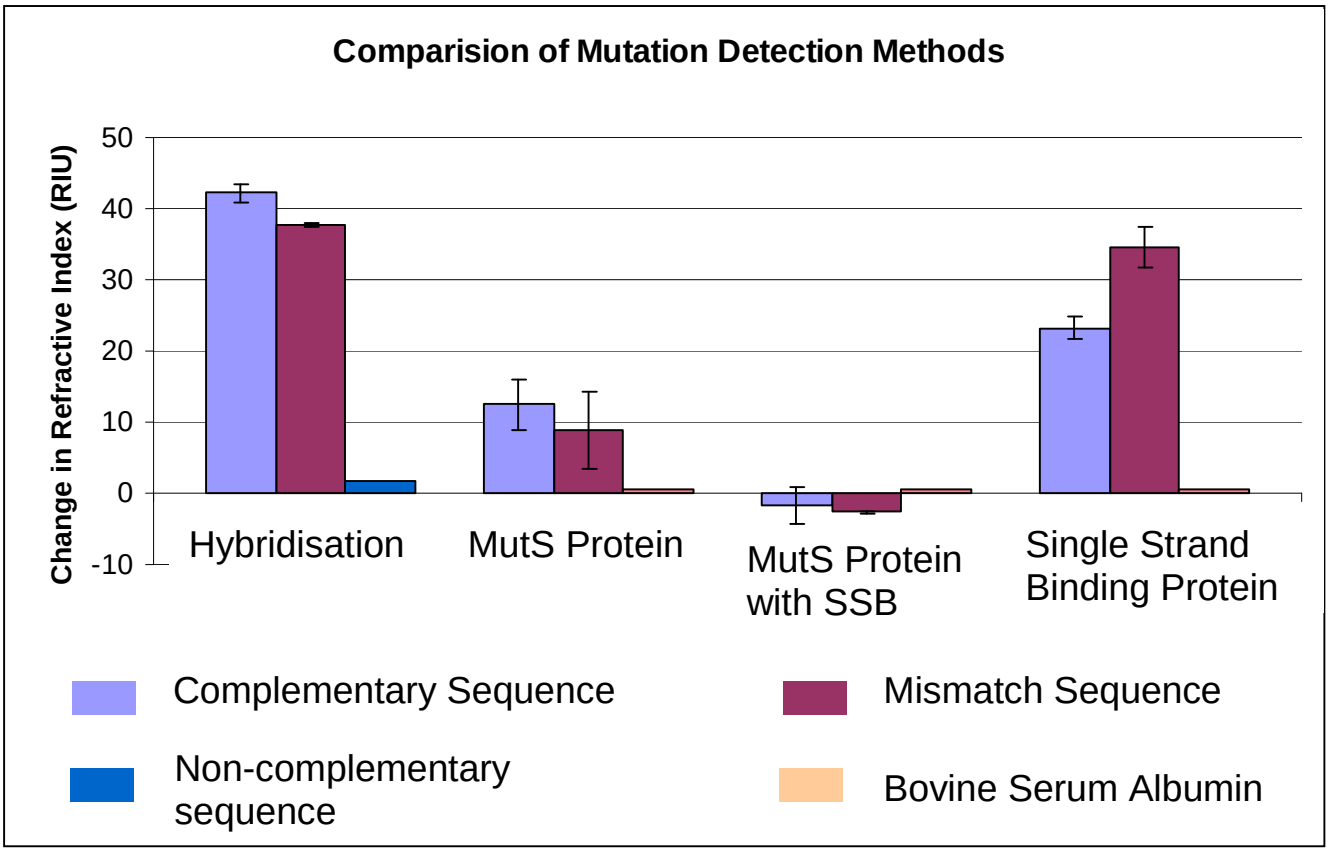

Figure 2: Comparison of the effectiveness of the mutation detection methods attempted in this study. Based on 4 hybridisation measurements, 2 measurements with MutS and MutS with SSB, and 4 measurements with SSB alone.

5. **References**

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