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1 Introduction

Cervical cancer has the fourth highest incidence of cancers in women worldwide [1] and incidence is highest in low income countries, where routine cervical screening programmes are mainly absent and diets are deficient in possible protective factors [2]. It is well established that the main risk factor for cervical cancer is infection with the high-risk human papilloma virus (HR-HPV) [3] and for this reason HPV screening has been introduced into cervical screening programmes around the globe [201]. The human papilloma virus consists of many strains with HPV-16 and HPV-18 being responsible for around 70% of cervical cancers [202], however not all women with HR-HPV infection develop cervical cancer but the persistence of certain high risk strains is a risk factor for precancerous cervical cell lesions (CIN) and therefore increases the risk of progression to cervical cancer [3, 4, 5]. As women with cervical cell abnormalities and persistent infection with HR-HPV have an increased risk of developing cervical cancer [4], early detection of cervical cell abnormalities and of persistence of the virus are central to cancer prevention and mortality reduction. There is therefore, much interest in the factors that influence the acquisition and the persistence of HR-HPV infection as well as in the classification of the severity of early stage precancerous cell lesions, as an understanding of these processes will inform strategies to reduce cervical cancer risk.

The mechanisms that support HPV persistence rather than clearance are not understood. HPV testing, while important in an assessment of cancer risk, does not predict whether the infection will be rapidly cleared or will persist. Monitoring for early stage cervical cell abnormalities requires scoring cell appearance by microscopy, which is time-consuming and has variable sensitivity. Changes that occur in the development of the cancer phenotype may include epigenetic modification to DNA, including methylation, and expression of genes important to the regulation of processes such as the cell cycle, DNA repair and apoptosis. Such changes in gene expression will be reflected in changes in both the cell proteome and metabolome and therefore, should be detectable by proteomic or metabolite profiling techniques.

Many metabolite profiling studies have concentrated on using Nuclear Magnetic Resonance, [6, 7] which is not practical as a routine technique in a clinical laboratory, or have concentrated on targeting specific metabolites [8] which involves a more complex analysis. Whilst targeting particular classes of compounds has value, such targeted analysis does not capture or provide a global picture of the most important discriminatory metabolites in the different cells. This means that some important metabolite changes and markers could easily be missed and therefore, particularly in the absence of specific hypotheses relating to mechanism underpinning HPV persistence, a non-targeted approach is more valuable.

The current screening and triage for cervical cancer risk uses HR-HPV infection and cytology to inform patient treatment. The most vulnerable women are those in whom the HR-HPV infection is likely to persist but have a low grade cellular abnormality, as these women are generally not treated but recalled 12 months after the initial HPV test. Not only is there a high loss to follow-up among this group but there is potential for progression of low grade lesions. For these reasons in this study we targeted HPV positive women with low grade lesions.

The aim here is to present a rapid metabolite screening method using Direct Injection Mass Spectrometry (DIMS) along with Principal Component Analysis (PCA)as tools to discriminate between cervical cell samples at different early-stage precancerous change, and between those samples from which HR-HPV virus is cleared and those in which it persists.

2 Materials and Methods

2.1 Sample History

The samples were provided by the Department of Oncology in the University of Sheffield Medical School. The samples were part of a wider study called the ARTISTIC trial (A Randomised Trial of HPV Testing in Primary Cervical Screening) [9] which involved routine cervical screening and HPV testing in 24,510 women aged between 20-64 years old. The primary aim of the ARTISTIC trial was to provide clear evidence on the costs, medical effects and psychosocial impact of adding HPV testing to cervical cytology screening.

At entry to the ARTISTIC trial cervical cell samples were collected from all women using liquid based cytology. All samples were examined for evidence of cervical cell abnormalities and HPV positivity and then stored at -80^{o}C until further use.

Usually the gold standard for new methods for cervical cancer screening and triage is histologically confirmed high grade dysplasia (CIN2+). Parameters for new methods include sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for CIN2+ with ROC curves used to define cut-offs for the test. CIN2+ is a surrogate marker for cervical cancer and HPV persistence is a surrogate marker for CIN2+. However for the purpose of our investigation we used a classification of abnormality according to cytology investigations, and only women with a classification of normal, borderline/mild and >= moderate abnormality were recruited [10].

For this study only the samples in the borderline and mild categories (with borderline being the least abnormal) were selected for analysis by metabolite profiling as these women would normally be recalled in 12 months' time. Samples included those from women who tested HR-HPV positive at 24 month follow up (cases), and women in whom no HR-HPV was detected at this point (controls). These samples were primarily selected for a study of epigenetic determinants of HR-HPV persistence [11] and surplus sample material was then used for the metabolite profiling described here.

2.2 Sample Preparation

160 samples were selected for metabolite analysis, based on the results of the cytology screen at baseline and HPV test at follow-up. All samples had a low-grade abnormality (borderline or mild) and tested positive for HR-HPV at baseline. Case samples were classed as women at baseline (the start of the trial) who were diagnosed with low-grade cervical neoplasia and who tested positive for HR-HPV infection and who were still HR-HPV positive 24 months later. Controls were women with the same diagnosis at baseline but who had cleared the infection and tested negative for HR-HPV 24 months later. In total there were 40 samples of each type which were selected at random: borderline case, borderline control, mild case and mild control.

HR-HPV classification was undertaken prior to this work by the ARTISTIC trial by using Hybrid Capture® 2 (hc2) according to the manufacturer's instructions [10]. Positive results were expressed in relative light units (RLUs) compared with a positive control containing 1pgml-1 of HPV DNA. The high-risk HPV types detected by the assay are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Samples were classified as HPV positive according to the manufacturer's instructions at the outset of the study, which was to use a positive cut off as 1 RLU/control. Persistence of HR-HPV was classified as the case samples which tested positive for the presence of HR-HPV at time 0 as well as at recall 24 months later.

The cell extracts for this study were prepared by authors HP and JF at the University of Sheffield Medical School [11] following the standard operating procedure for rapid purification of DNA from cell samples supplied with a standard QIAamp DNA Mini Kit, purchased from Qiagen (part number 51304). The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from tissues, swabs, CSF, blood, body fluids, or washed cells from urine use an optimised mix of buffers and enzymes. The supernatant which would normally be discarded after DNA extraction was retained and stored at -20°C, in this case, for the metabolite profile analysis.

2.3 Direct Injection Mass Spectrometer Analysis

All analysis was carried out by directly injecting each sample into a Waters Micromass Liquid Chromatograph Time of Flight mass spectrometer (LCT) (Waters, Manchester, UK). The mass spectrometer was operated in both positive and negative modes in order to analyse as many metabolites as possible, however only data from the positive mode analysis will be discussed here. The conditions for the analysis were capillary 3000V, extraction cone 5V, sample cone 15V, RF lens 75V with source and desolvation temperatures at 100°C and 150°C respectively. Spectra were collected in continuum mode over the mass range 75-800amu at a rate of one scan per 0.5sec with an interscan delay of 0.1sec.

Due to the high concentration of buffer in the samples, matrix effects were minimised by diluting with 1:500 v/v with high purity water:methanol (50:50 v/v) before analysis. This dilution was found to be optimal for sensitivity, reduced ion suppression effects and ease of analysis.

The sample set of 160 samples, 40 of each of the 4 types, were arranged as a double blind experiment as the identity of the samples was unknown to the analyst, and the samples were all randomised for analysis.

To show the robustness of this technique a small set of the samples were re-analysed in positive mode on a portable Advion CMS compact mass spectrometer. The conditions for this analysis were ESI voltage 3500V, capillary 180V, source voltage offset 20V, source voltage span 30V and capillary and source gas temperatures 250^{o}C and 200^{o}C respectively. Spectra were collected over the range 50-800amu at a rate of one scan per 0.38sec with an interscan delay of 0.1msec.

2.4 Data Processing and Multivariate Analysis

Three replicate total ion chromatograms (TIC) of each individual sample were obtained to enable noise reduction according to the procedure defined by Overy and colleagues [12]. For each mass the summed intensity from the TIC was averaged to minimise variation and the data for each sample were normalised to the TIC for each biological replicate. In this case the data were rounded into 0.4amu bins to minimise the number of peaks. The binned data sets were saved as metabolite profiles in text file format.

The data were exported into SIMCA-P (Umetrics) to create PCA and OPLS-DA plots for the data analysis and the significantly changing bins were tabulated along with standard errors calculated from the standard deviation of the mean.

3 Results and Discussion

Direct injection mass spectrometry (DIMS) was used without any clean-up or chromatography step before analysis, for reasons of simplicity and speed. Each analysis took only 2 minutes using this approach and the samples required no preparation apart from a simple dilution. This made the procedure routine and with the current introduction of portable mass spectrometers the procedure

would be very quick and easy to run in a clinical laboratory, even for staff who are unskilled in mass spectrometry. The DNA extraction step removing large molecules could be further simplified.

A challenge with this analysis was overcoming the problems of dealing with the difficult chemical matrix of the samples and possible suppression of ions due to the buffers used for the DNA extraction method. A large dilution had to be used (1:500) but this allowed the samples to be analysed routinely and did not significantly affect the sensitivity or the findings of the analysis as the overall number of peaks detected did not significantly change with increasing dilution.

Initially all samples acquired in the same analysis mode were analysed together by PCA (data not shown). The case mild samples exhibited a clear cluster away from control mild but also away from borderline samples. Although mild cases and controls showed reasonably distinct clustering the borderline case samples did not show a tight cluster, rather they appeared to divide into two clusters. The mild and borderline samples were therefore plotted in separate PCA plots (figure 1).

Fig. 1 PCA Scores Plot for mild samples only (a) and borderline sample only (b). The numbers refer to individual sample ID. Samples were analysed by direct injection mass spectrometry, mass intensities normalised to total ion count and the resulting data examined in a PCA analysis with SIMCA-P.

The scores plot for the mild samples (figure 1a) shows a very clear difference between the case and control samples suggesting that the ability to clear the HPV infection is reflected in an altered metabolome.

Analysis of the borderline samples separately (figure 1b) reveals that, although there is some clustering of cases as distinct from controls neither classification shows a tight cluster. This contrasts with the clear clustering observed for the mild samples. The cytological classification of borderline which was used for the samples in the Artistic trial indicated that there was some level of abnormality but insufficient to classify the samples as mild. Therefore it is possible that this cytological classification of cellular abnormalities may not be particularly distinct and includes a high level of false positives [13, 14]. The division into two groups by metabolite profiling may reflect this

by distinguishing between those patients that clear the virus and those that do not and this different metabolite profile may not be associated with a low level of cell abnormality.

From the loadings plot (figure 2) corresponding to scores plot for the mild samples (figure 1a) the metabolite bins 119, 155, 157 and 385.4 (seen in the right hand side of fig. 2) strongly influenced the separation of the classes, with the metabolite bin 385.4 having the strongest effect. At this stage the identification of the metabolites in the individual bins was not determined, as the aim of the study was to investigate whether the sample type contained a specific metabolite profile. The loadings plot for the borderline samples (figure not shown) shows that the 385.4 bin again strongly influences the separation seen in the scores plot.

Fig. 2 Loadings plot to show mass bins responsible for separation in PCA scores plot (Fig. 1a) for mild samples in positive mode. The labels refer to individual mass unit bins.

Further analysis of the data showed that although other bins were responsible for the separation in the PCA plot none was as striking, between cases and controls for both borderline and mild, as the 385.4 bin. No identification of the metabolites present was undertaken in this study as it was intended to be a rapid screening method although identification could be done using MS-MS.

The data collected from the analysis on the portable Advion CMS were treated in exactly the same way as the data collected from the Waters LCT and although the mass resolution and sensitivity of the two instruments is different the same clustering patterns were seen in the PCA scores plots (figure 3). The PCA loadings plots also showed the same patterns with the same major 385.4 mass bin responsible for the separation of the classes seen in the scores plots (data not shown).

Fig. 3 PCA scores plot generated from data collected by direct injection on an Advion portable compact mass spectrometer (CMS) for mild samples only in positive mode. Mass intensities normalised to total ion count and the resulting data examined in a PCA analysis with SIMCA-P. The numbers refer to individual sample ID.

The findings are important because they suggest that metabolite profiling could offer an accurate, and time-effective tool for identifying women at increased risk of HPV persistence.Current screening for cervical cancer risk relies, as a first step, on a cytological assessment which can be difficult to classify at the low grade end of cervical cell abnormality and currently there is no screening tool for predicting HPV persistence. Metabolite profiling therefore has the potential to improve existing screening methods for cell abnormality and to supplement information about HPV positivity; the critically important risk factor for cervical cancer.

Despite the differences in the likelihood of different HR-HPV types persisting, metabolite profiling was able to distinguish between cases and controls, suggesting that the metabolite profile was independent of strain of HPV and any changes in metabolism was due to the presence of the virus and not the individual type. The tool therefore would be useful even when HR-HPV type was not known or not factored into the analysis.

Additionally, metabolite profiling was able to distinguish levels of low grade cell abnormality, which is difficult to do using conventional microscopic screening. This has the potential to reduce the risk of mis-classification thereby minimising the costly clinic recall for women providing a classification of 'indeterminate'. Metabolite profiles could also reveal new targets for pharmaceutical intervention for influencing HPV persistence [15, 16].

4 Concluding Remarks

A mass spectrometer direct injection method for the analysis of a large number of biological replicates has been shown to be highly effective in discriminating samples on the basis of stage of low grade cervical cell abnormality, and also for HPV persistence. The ability to clearly predict HPV

persistence was greater for samples with a mild classification, which may reflect a more homogeneous sample than for those classified as borderline, which likely contain false positives. The ability to discriminate between women who need more intensive monitoring and those who do not could greatly benefit health care systems worldwide. Type of HPV strain was not taken into consideration for this study as it has been previously shown that type of strain has no effect on clearance of the virus [17]. Also age was not taken into consideration even though it is considered to be a factor influencing HR-HPV persistence because age was found not to be significantly different between cases and controls in the original study (30.1±8.9 vs 31.6±9.5years) [11]. With the increased portability of mass spectrometers and therefore the reduced cost this technique becomes more affordable for low and middle income countries. The method is fast, uses a very small amount of sample, and could readily be integrated into existing care protocols in different settings without any added complex sample preparation [18].

5 Future Perspectives

Current screening and triage for cervical cancer risk rely on HR-HPV testing and a cytological assessment of cervical cell abnormality, which is difficult to classify at the low grade end of cervical cell abnormality. This is important because, given that there is no diagnostic tool for HR-HPV persistence, women with HR-HPV infection and low grade abnormality or normal cells, are generally not treated but recalled at a later date, with an associated loss to follow up. Metabolite profiling could become an integral part of screening and triage for cervical cancer risk, particularly in low and middle income countries, where significant progress is being made to introduce screening programs, involving low cost HPV testing and cytological examination.

As the use of MS is increasing in clinical laboratories, due to improved knowledge, simplification of technology, changing perceptions and training, this novel and rapid method has real potential value for the prediction of HPV persistence and offers considerable efficiency savings in the prevention of this cancer type since patient recall could be targeted effectively to those most at risk.

6 Executive Summary

Background

- Screening and diagnosis for cervical cancer requires time consuming and labour intensive cytology and in some countries also includes identification of the presence of high risk human papillomavirus (HR-HPV). With the advent of rapid metabolic profiling techniques and the introduction of portable mass spectrometers we examined whether cells distinguished by their cytology and the presence of a persistent HPV infection, could be easily differentiated by their metabolite profile.

Experimental

- Direct injection electrospray mass spectrometry (DIMS) was used for rapid metabolite profiling of cervical cell samples in a non-targeted double blind experiment.
- Cell extracts were prepared using a routine DNA extraction procedure and the resulting supernatant, normally discarded, was analysed by mass spectrometry.
- Data were interpreted using principal component analysis (PCA).

Results and Discussion

- The samples analysed had been collected from women infected with high risk human papillomavirus (HR-HPV) and diagnosed with one of two grades of cervical cytology and exhibiting either HPV persistence or clearance.
- The metabolite profiles obtained clearly differentiated samples with different grades of cell abnormality and HPV persistence or clearance.

Conclusion

- The results indicate strongly that a simple metabolite extraction procedure and mass spectrometric profiling method could be used to rapidly identify women at increased risk of HPV persistence..

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Compliance with Ethical Standards

Ethics approval for use of samples from the ARTISTIC study was obtained from Multicentre Research Ethics Committee, North West, UK (MREC 00/8/30)

Keywords

Cervical Cancer

HPV

Mass Spectrometry

Metabolomics

Metabolite Profiling