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ORIGINAL ARTICLE

The Value of a Novel Panel of Cervical Cancer Biomarkers for Triage of HPV Positive Patients and for Detecting Disease Progression

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Abstract In the era of primary vaccination against HPV and at the beginning of the low prevalence of cervical lesions, introduction of screening methods that can distinguish between low- and high-grade lesions is necessary in order to maintain the positive predictive value of screening. This case-control study included 562 women who attended cervical screening or were referred for colposcopy and 140 disease free controls, confirmed by histology and/or cytology. The cases were stratified by age. Using routine exfoliated liquid based cytological samples RT-PCR measurements of biomarker genes, high-risk HPV testing and liquid based cytology were performed and used to evaluate different testing protocols including sets of genes/tests with different test cut-offs for the diagnostic panels. Three new panels of cellular biomarkers for improved triage of hrHPV positive women (diagnostic panel) and for prognostic assessment of CIN lesions were proposed. The diagnostic panel (PIK3AP1, TP63 and DSG3) has the potential to distinguish cytologically normal hrHPV+ women from hrHPV+ women with CIN2+. The prognostic gene panels (KRT78, MUC5AC, BPIFB1 and CXCL13, TP63, DSG3) have the ability to differentiate hrHPV+ CIN1 and carcinoma cases. The diagnostic triage panel showed good likelihood ratios for all age groups. The panel showed age-unrelated performance and even better diagnostic value under age 30, a unique feature among the established cervical triage tests. The prognostic gene-panels demonstrated good discriminatory power and oncogenic, anti-oncogenic grouping of genes. The study highlights the potential for the gene expression panels to be used for diagnostic triage and lesion prognostics in cervical cancer screening.

Keywords Human papillomavirus · Cervical neoplasia · Case-control study · Cellular biomarker, biomarker, cervical cancer, carcinogenesis

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Introduction

Global cervical cancer incidence has increased from 378.000 (256.000–489.000) cases per year in 1980 to 454.000 (318.000–620.000) cases per year in 2010, representing a

0.6 % annual rate of increase. Death rates of cervical cancer are decreasing, however this disease still remains a major cause of cancer deaths in women, with 200.000 deaths in 2010, from which 46.000 were patients aged between 15 and 49 years in developing countries [1, 2]. The introduction of cervical screening programmes based on the PAP test significantly reduced the incidence of cervical cancer, however there still remain some issues in relation to the sensitivity and specificity of cytology. Human Papillomavirus (HPV) is the single most important aetiological agent in the pathogenesis of cervical cancer and pre-cancer. Prophylactic vaccination against HPV16/18 (and, in some instances, the low-risk types HPV6/11) has been introduced as a primary cervical cancer prevention strategy in many countries [3]. Vaccination should reduce the incidence of cervical disease, however it will present new challenges to current screening approaches.

HPV testing plays an important role in cervical cancer prevention in both the pre- and post-HPV vaccination era by means of [a] being a primary screening tool, [b] ensuring the triage of women with low-grade cytological abnormalities and [c] providing follow-up of women treated for cervical intraepithelial neoplasia [CIN]. Several randomised control trials demonstrate the potential for primary screening with HPV DNA testing compared to cytology. However, while HPV DNA testing is more sensitive than cytology for identifying women with CIN2+, the specificity is lower [4]. The key challenge with HPV primary screening is to find the optimal balance between sensitivity and specificity and to avoid large numbers of unnecessary follow-up examinations of HPV-positive women.

A number of protein biomarkers have already been tested and validated for the identification of dysplastic cells in cervical smear specimens and therefore have the potential to enhance and improve current cervical screening performance. These markers include, but are not limited to HPV E6/E7mRNA, HPV L1, p16INK4a, TOPO2A and MCM2. These protein biomarkers might have value in cervical screening, however it is much more likely, due to their resource intensive characteristics, that they would be used as triage tests to confirm cytology diagnosis. Protein biomarkers also have a role in reduction of inter-observer bias of morphological diagnosis [5].

Recently, attention has focused on methylated genes of cervical lesions. Methylation studies of a CpG island within the p16INK4a exon1α have been inconclusive despite the association of methylated genes with a variety of malignant tumors, such as non-small cell lung cancer, or pancreatic cancer [6, 7]. Henken at al. have identified several grade-related methylation events, which partially overlap and confirm these genes (CADM1 and MAL) [8]. The methylation biomarkers have been extended to other viral and cellular genes and have been reviewed recently [9].

The aim of the present study was to evaluate the neglected mRNA biomarkers for their effectiveness in distinguishing cytologically/histologically normal women from those with high grade CIN, and in identifying the lesions most likely to progress. The aim includes the exploration of mRNA biomarkers as new triaging diagnostic tools for HPV+ women and the definition of a new set of the next-tier, cervical pre-cancer prognostic biomarkers. This was achieved in a case control cohort study consisting of women presenting for routine cervical screening and a subgroup of women referred for colposcopy to investigate their abnormal smears.

Materials and Methods

Cell-lines, Biological Specimens

Cell lines (HeLa, C33A) were obtained from ATCC and RNA extracted from cervical cancer specimens was obtained from the Ambion respository (First Choice Tumour/Normal Adjacent Cervix Total RNA). CIN3 tissue samples with corresponding healthy tissue and cervical carcinoma clinical samples as well as normal cervical epithelial tissues were purchased from BioServe (Beltsville, USA) for the microarray studies.

Study Population

Our study population involved 3 groups. Group 1 consisted of a cohort of women ($n = 90$) attending the colposcopy clinic at the Coombe Women and Infants University Hospital, Dublin, Ireland for investigations following an abnormal liquid based cytology (LBC, PreservCyt, Hologic) result. These women were recruited through CERVIVA, The Irish Cervical Screening Research Consortium (www.cerviva.ie). Cervical smear samples from patients referred for colposcopy with an abnormal smear were obtained prior to the colposcopic procedure. Histological results from biopsy taken during colposcopy were recorded and made available for the study. Group 2 comprised a total of 472 patients who underwent surgical conization (due to various reasons including prior high grade cervical cytology), both loop and knife, providing histology samples. Prior to the procedure, liquid based cytology (LBC, PreservCyt, Hologic) samples were taken. Group 3 contained liquid based cervical cytology samples from 140 consecutively enrolled patients who attended a routine population based screening in Hungary, with no indication of previous cervical disease. Women in Groups 2 and 3 were enrolled in HPV_SCREEN multicentre (7 centres) clinical study and KTI121128 KMR_BIOMARKER study in Hungary. After stratification of the colposcopic referral samples according to the histological diagnosis, 273 CIN2+ cases (including 31 CIN1/2, 65 CIN2, 38 CIN2/3, 114 CIN3 and 25 carcinomas) and 94 CIN1 and 335 cytology and/or histology negative cases were identified. The

cases were further categorized by HR HPV status (see below), resulting in 389 HR HPV positive and 308 HR HPV negative cases, and 5 cases were excluded due to the lack of HPV test results (HR HPV positivity for Group 1: 83.3 %, Group 2: 61.9 % and Group 3: 15.7 %, respectively). In the absence of a large screening population and following the recommendations of Arbyn et al. [10], diagnostic studies were performed in a clinical setting where all women were submitted to verification by the reference standard (histology or cytology). Written informed consent was obtained from all participants. The study was anonymous so that only investigators had access to full clinical data. Laboratory personnel had no information about the study participants and were blinded to case–control status.

Biomarker Genes

Genes were pre-selected on the basis of expression studies in cell lines and cervical cancer and normal cervical tissues. RNA was extracted using Trizol from cell lines (HeLa, C33A) and the purchased biobanked cervical cancer (Ambion) and CIN3 and corresponding healthy tissue (BioServe) that had been snap frozen within 30 min of arterial ligation. In brief, cDNA array expression levels were obtained using LifeTech (ABI1700 Chemiluminescent Microarray Analyser, Waltham, USA) and were evaluated for detection of 191 differentially over-expressed genes (see Supplemental Material). These genes were validated on 384 Wells TaqMan Low Density Arrays according to the procedures given below using clinical specimens (n = 143 from Group 2 and Group 3). On the basis of highest covariation (see below) with the disease status, 17 biomarker genes were selected for further evaluation (CYP24A1, DSG3, LGALS7, IL36RN, BPIFB1, IL1RN, PITX2, RTKN2, PIK3AP1, KRT78, CXCL13, EREG, TP63, KLK8, MUC5AC, SERPINB7, CYP2C18).

HPV Testing and Determination of Biomarker Gene Expression

Liquid-based cytology (LBC) specimens were transported between 4 and 8 °C before DNA and RNA isolation. DNA was isolated using AmpliLute Liquid Media Extraction Kit (Roche, Basel, Switzerland), RNA was isolated by M48 QiaRobot and Total Nucleic Acid Kit (Qiagen, Hilden, Germany) for Group 1 and RecoverAll Total Nucleic Acid Isolation Kit (LifeTech, Waltham, USA) for Group 2 and Group 3. Reverse transcription was carried out with TaqMan Reverse Transcription Reagents Kit (LifeTech) with random hexamers. Diagnostic HPV assays were carried out using GenoID's Full Spectrum HPV Amplification and Detection System (FS) (n = 697). (Five cases did not have determined status (ND) and were treated accordingly) [11]. All assays were performed according to the manufacturer's instructions. 384 Wells TaqMan Low Density Arrays real-time PCR amplifications were carried out using 7900HT Fast Real-Time PCR System (LifeTech), on custom-designed 384-wells 7900HT Gene Expression Micro Fluidic Cards.

Statistical Analysis

To select the best performing biomarker genes from the 191 primary biomarkers according to the biomarker status, a complex algorithm was devised using shallow tree classificator, which was averaged by boosting and was used to input bagging algorithm to suppress over-fitting, using the data mining tool Weka.

The selected 17 genes were evaluated against all groups to determine a negative and a positive cut-off Ct value for each. According to the comparison between measured values for the genes and the cut-off values, each gene was assigned a score of 1, 0 or −1, based on whether their numerical values (Ct values) were less than, between or greater than the negative and positive cut-off values. The allocated scores were added together to provide the total sum of the scores. A pre-determined threshold value of the total sum was applied to differentiate the test positive and test negative cases. To demonstrate the diagnostic behaviour of different sets of biomarker genes special ROC curves were constructed, using the combinations of the 17 genes, with different thresholds calculated and plotted against sensitivity and specificity. Results were evaluated according to basic contingency statistics including Likelihood Ratios and Chi-squared Mantel-Haenszel.

For further details of calculations including details on how the images were generated, see the figure and table captions and the computational details are available from the authors.

Results

Primary Selection of Biomarker Genes

Expression profiles of normal tissues paired with squamous carcinomas and adjacent normal tissues (n = 3), a CIN3 case (n = 1) along with a cervical carcinoma cell-line (HeLa), and C33A as a HPV negative immortalized control were used to select the most promising biomarker genes. On the basis of these gene expression studies, 191 genes were selected and verified by reverse transcription TaqMan assays in 384 Wells TaqMan Low Density Arrays real-time PCR amplifications in duplicates (including 18S RNA as house-keeping gene and LCE3D as the tissue-specific normalisation gene). The quality of the mRNA of the samples were accessed by measuring of the expression of 18S gene and other housekeeping genes, if

the 18S Ct value was greater than 34 (samples were excluded on the basis of this measures). The effect of storage time was also accessed and no significant relationship was found between storage time and 18S expression, indicating good preservation of the mRNA content of the samples over time (up to 2 months). The resulting expression values were used to calculate concordance between gene expression and clinical outcome using the data mining tool Weka [12]. To classify samples according to their biomarker status, an algorithm was devised using the gene specific housekeeping normalized Ct values (shallow tree classificator). The resulting panel of genes showed strong clustering with CIN2+ cases (Fig. 1). The clusterogram of histology confirmed cervical LBC specimens (columns, n = 702) based on housekeeping gene (LCE3D), ΔΔCt normalized TaqMan Low Density Array revealed the results of preselected biomarker genes (rows, n = 17), (Fig. 1). Visual inspection of the clusterogram made possible the identification of subclusters with preferentially low or high expression of biomarker genes. SC1 (sub-cluster 1) had 29.3 % of cytology negative sine morbo (SM) cases and 70.7 % of CIN2+ (cervical intraepithelial neoplasia 2 or higher) cases, similarly SC2 had 38.3 % of SM and 61.7 % of CIN2+, and SC3 had 64.4 % of SM and 35.6 % of CIN2+ cases, respectively.

Identification of Biomarker Gene-Expression Differences According to Pathological Categories

Principal component analysis (PCA) of histology confirmed cervical LBC specimens (n = 702) based on normalized TaqMan Low Density Array results of pre-selected biomarker genes (n = 17) was carried out to reduce the dimensionality of the gene-expression measurements (Fig. 2). The shape of the hulls and the direction of the contribution vectors are compatible with the notion that the biological variables represented by the first two principal components have near similar effects and that most of the genes are contributors in both variables. The positive contributing biomarkers (pointing toward carcinoma centroid) were found to be RTKN2, DSG3, PITX2, CXCL13, CYP24A1, BPIFB1, CYP2C18, MUC5AC, LGALS7 and TP63, while the negatively contributing genes were IL36RN, KRT78, EREG, IL1RN, SERPINB7, KLK8 and PIK3AP1.

The convex hulls (Fig. 2, Panel a) and the centroids showed no clear separation between normal and CINs/CC cases based on the gene-expressions of the 17 genes, however many of the CIN2 cases were well-separated from all other categories. This indicates a unique behaviour and the displacement of centroids confers a transitional nature of a subset of

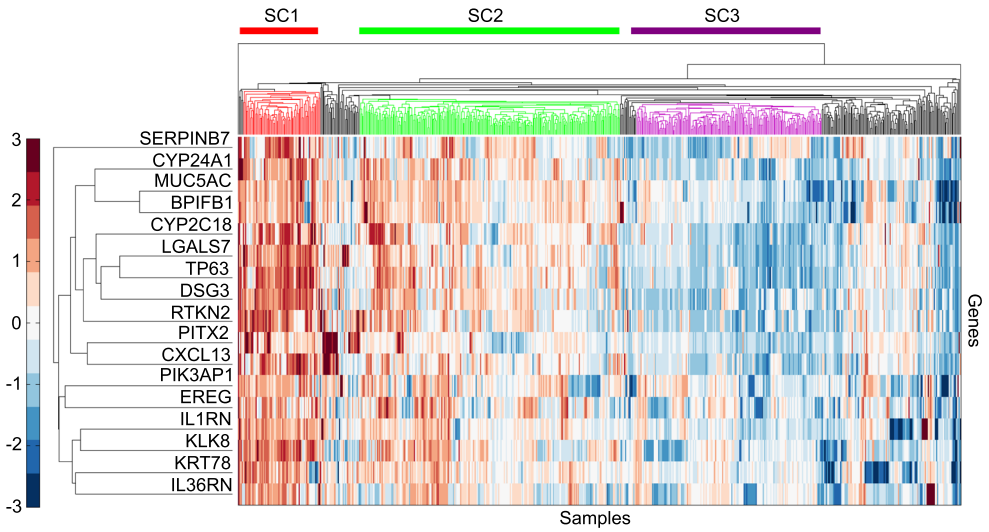

Fig. 1 Clusterogram (hierarchical clustering, dendrogram and heat map; standardized according to the genes to have zero mean and standard deviation equal to one; pairwise distance metric is euclidean for both axis; hierarchical cluster tree linkage method is averaging) of histology confirmed cervical scraping specimens (columns, n = 702) is shown based on housekeeping gene (LCE3D) ΔΔCt normalized TaqMan Low Density Array results of pre-selected biomarkers genes (rows, n = 17). As the test normality and variance homogeneity of the biomarker gene expressions failed (Jarque-Bera normality test failed at $p < 0.01$ for 15 of 17 genes and according to the Leven's Test $p < 0.05$, 4 of 17 genes have inhomogeneous variances) the non-parametric Kruskal-Wallis ANOVA was applied to group the cases according to CIN2+ and negative diagnosis categories and the false-discovery adjusted p-values were calculated ($0 < q < =0.006$, except for IL36FN, BPIFB1 and KRT78, which were not significant at $p < 0.05$). The sub-clusterograms include visually chosen samples, which preferably have high or low biomarker gene expressions. SC1 (sub-cluster 1) has 29.3 % of negative sine morbo (SM) cases and 70.7 % of CIN2+ (cervical intraepithelial neoplasia 2 or higher) cases, similarly, SC2 has 38.3 % of SM and 61.7 % of CIN2+, and SC3 has 64.4 % of SM and 35.6 % of CIN2+ cases, respectively. As the analytic distribution of the statistic is unknown, to validate the arbitrary selection of sub-clusters the Aslan & Zech's test based on an analogy to statistical energy was implemented [42] and bootstrapping (n = 100) was used to calculate similarity of SC1, SC2 and SC3 to randomly chosen, same-sized sub-clusters of the clusterogram (no similarity found). See computational details in Supplement Material

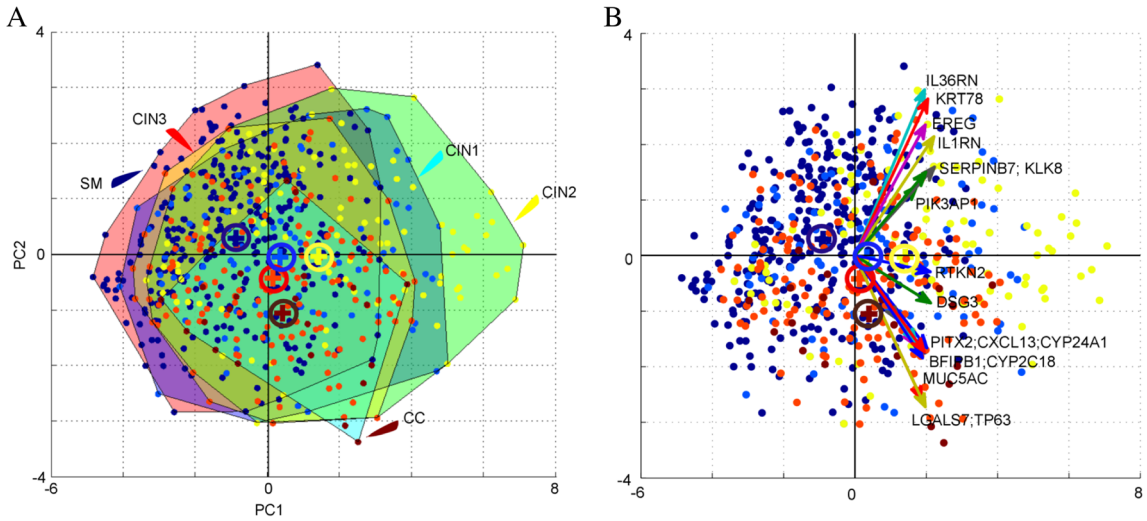

Fig. 2 Principal component analysis (PCA) of histology confirmed cervical scraping specimens (n = 702) based on normalized TaqMan Low Density Array results of pre-selected biomarker genes (n = 17). The normalization was done by mapping each sample's means to 0 and deviations to unity. Negative, cytology negative, sine morbo (SM) cases are shown as blue dots (n = 335) and similarly, CIN1 cases are cyan (n = 125), CIN2 are yellow (n = 134), CIN3 are red (n = 152) and carcinoma cases (CC, n = 25) are brown dots. Some cases were counted in two pathological categories, according to the diagnosis made originally (CIN1 + &CIN2+, n = 31 and CIN2 + &CIN3+, n = 38). **a** The convex hulls of pathological categories are indicated according to the first two principal components (PC1, PC2) of PCA (percent of explained variance: 53.9 %) showing the occupied space of the pathological categories. Centroids were calculated for each category and the convex hulls were constructed using the 95 % of cases that were the closest (euclidean distance) to the (all data) categorical centroid to eliminate extreme values. Centroids are circled crosses and are shown in the colour of the matching pathological categories. **b** Contribution vectors of biomarker genes according to the first two principal components of PCA, cases are indicated as described above, gene names are shown, vector colours are arbitrary

CIN2 cases, as both CIN1 and CIN3 cases were clustered together, while the CIN2 centroid was found to be isolated. Nevertheless, a significant portion of SM/negative cases was also in clear isolation from the pre-cancer cases, however the overlaps were large.

Diagnostic Performance of Diagnostic Gene Panel in High-risk HPV Positive Population

Threesome sets of biomarker genes were evaluated at various thresholds (from −3 to 3) and analysed for their ability to differentiate normal (n = 83) and CIN2+ (n = 247) cases in the high-risk HPV positive population. For each gene, a negative reference/cut-off value and a positive reference/cut-off value (mean of LCE3D normalised expression of negative and positive cases for each gene) were determined (see Materials and Methods). The LCE3D normalized Ct values of gene expression determined by TaqMan Low Density Array assays were compared with these cut-offs enabling a score to be determined for each sample. Cases were given a positive test result if the total sum value of scores was higher than the given threshold value. All gene and threshold combinations were plotted according to their sensitivity and specificity. Figure 3a shows the discriminatory performance of three-gene sets (diagnostic panel) between normal and CIN2+ cases in the HR HPV+ population. A significant portion of the sets performed better than a random guess, as the majority of the points of sets were positioned above the diagonal line (from lower left to upper right corners). This behaviour indicates that all the biomarker genes are contributors to the discrimination of normal and CIN2+ cases. Some of the genes showed very high specificities indicating their high potential diagnostic value. The performance values of the best performing panel of genes with the best threshold (PIK3AP1, TP63 and DSG3 evaluated at threshold value of 0) are shown in Table 1. In the HPV positive population of cases this panel exhibited a sensitivity and specificity of 67.5 % and 68.8 %, respectively. As +LR above 2.5 and -LR below 0.25 are considered as an excellent performance for a diagnostic test, the resulting values indicate good test performance (see Table 1).

Age-Unrelated diagnostic Performance of Differentiation of Normal and CIN2 or Worse Cases in High-Risk HPV Positive Population

Age stratified results of the diagnostic, triage panel were calculated and the contingency tables were constructed (see Table 2), using the best set of genes (PIK3AP1, TP63 and DSG3). No significant differences were observed between the 2 contingency tables (p = 0.21), (Chi Square 4.5), (4 × 2 contingency, homogeneity testing), indicating similar age-unrelated discriminatory power for normal and CIN2+ cases. This is contrary to the behaviour of other cervical pre-cancer diagnostic tests including high-risk HPV testing, p16 immunostaining and methylation markers [13].

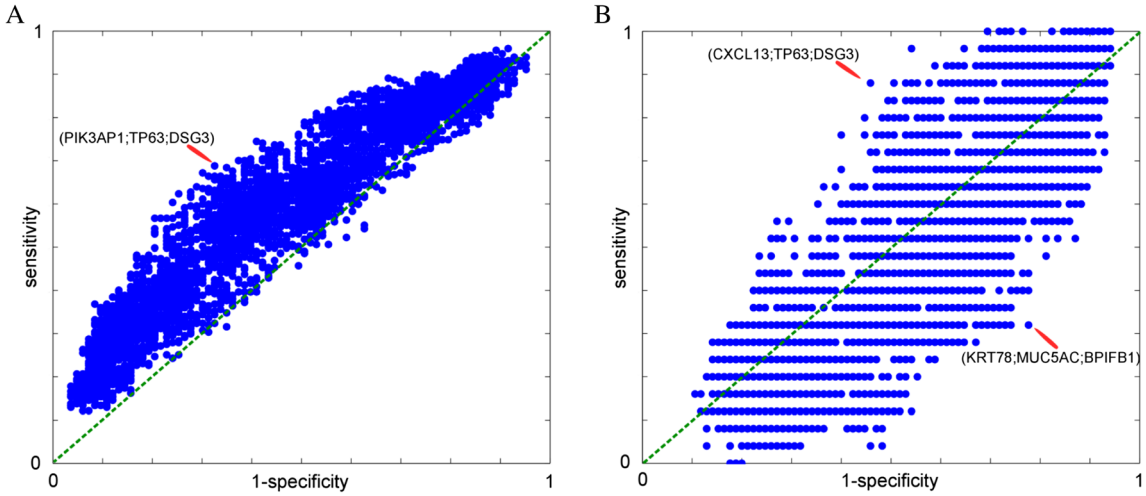

Fig. 3 shows the ROC plot of the results of discrimination of normal and CIN2+ cases and the results of CIN1 – carcinoma discrimination. **a** Sets of biomarker genes (all possible combinations of three genes of the 17 gene panel) were evaluated at different thresholds, see Materials and Methods. The samples were histologically confirmed cervical scraping specimens (HR HPV+ cytology negatives, n = 83; HR HPV+ CIN2+, n = 247). Test positivity and negativity were calculated using LCE3D normalized TaqMan Low Density Array results; in brief, the positive and negative cut-off Ct values were calculated for each gene and the individual scores were calculated for each gene comparing the individual (sample and gene specific) Ct to the cut-offs. A value of −1, 0 or 1 was assigned and the scores were summed. The cases were evaluated at different thresholds of score. The gene set of PIK3AP1, TP63 and DSG3 at threshold of 0 is indicated. Sensitivity and specificity for the detection of HPV+ CIN2+ were 67.5 % and 68.8 %, respectively. **b** Sets of biomarker genes (all possible sets of three genes of 17 genes) were evaluated at different thresholds, see Materials and Methods. The samples were histologically confirmed cervical scraping specimens (HR HPV+ CIN1, n = 85; CC, n = 25). Test positivity and negativity were calculated using LCE3D normalized TaqMan Low Density Array results; in brief, the positive and negative cut-off Ct values were calculated for each gene and the individual scores were calculated for each gene comparing the individual (sample and gene specific) Ct to the cut-offs, values of −1, 0 or 1 were assigned and the scores were summed. The cases were evaluated at different thresholds of score. The gene sets of KRT78, MUC5AC and BPIFB at threshold of −1, as well as of CXCL13, TP63 and DSG3 at threshold of 0 are indicated. The sensitivities (s) and specificities (sp) for the detection of CIN1 (negative discriminator) are sp.: 77.6 % and s: 68.0 % and for the detection of CC (positive discriminator) sp.: 54.1 % and s: 88.0 %

Prognostic Gene Panel to Differentiate CIN1 and Carcinoma Cases

Figure 3b shows the discrimination ability of the three-gene sets between CIN1 (n = 151) and carcinoma (n = 30) cases with various threshold permutations (from −3 to 3). The LCE3D normalized Ct values of gene expressions were analysed as for the diagnostic gene panel. A significant portion of gene sets was better than a random guess, indicating that all biomarker genes contributed to the discrimination of cervical CIN1 pre-cancer and cancer, and that each of them had a significant positive or negative effect. The performance values of the best performing positive contributory panel of genes with the best performing threshold are shown in Table 3. Since the ROC curves were almost symmetrical along the non-discriminatory diagonal line, the best positive and negative contributor three-gene sets were determined; the gene panel consisting of CXCL13, TP63 and DSG3 and the gene set of KRT78, MUC5AC and BPIFB, respectively (see Table 3 for details). The sensitivity (77.6 %) and specificity (68.0 %) of KRT78, MUC5AC and BPIFB (the negative discriminator) were determined in regard to the detection of CIN1 and the sensitivity (54.1 %) and specificity (88.0 %) of CXCL13, TP63 and DSG3 (the positive discriminator) were identified in relation to the detection of carcinoma. These values indicated highly distinctive diagnostic behaviour, moreover using the positive discriminatory panel the likelihood of test positivity increased according to the CIN grade with a + LR of 46 % for CIN1 (n = 85), 63 % for CIN2 (n = 122), 55 % for CIN3 (n = 138) and 88 % for carcinoma (n = 25).

Table 1 Contingency calculations of the best gene panel for the discrimination of normal and CIN2 or worse HR HPV positive: PIK3AP1, TP63 and DSG3 (evaluated at threshold value of 0) for all ages. Positive Likelihood Ratio (+LR) = Sensitivity / (1 - Specificity): 2.116 (CI95% 1.552–3.003) and the Negative Likelihood Ratio (−LR) = (1 - Sensitivity) / Specificity 0.462 (CI95% 0.371–0.596). Chi Square - Mantel-Haenszel 33.913, p = 0.001

Diagnostic biomarker set	Diagnosis positive (CIN2+)	Diagnosis negative (Cytology Negative)	Totals
Test Positive	170	27	197
Test Negative	77	56	133
Totals	247	83	330

Discussion

The genes selected for this case-control study are involved in a wide range of cellular processes and many of them have not

Table 2 Contingency calculations of the best gene panel for the discrimination of normal and CIN2 or worse HR HPV positive: PIK3AP1, TP63 and DSG3 (evaluated at threshold value of 0) for all ages. A. for women aged 30 years or older, Positive Likelihood Ratio (+LR) = Sensitivity / (1 - Specificity): 1.773 (CI95% 1.260–2.646) and Negative Likelihood Ratio (−LR) = (1 - Sensitivity) / Specificity 0.536 (CI95% 0.409–0.743). Chi Square - Mantel-Haenszel 14.812, $p = 0.001$. B. for women younger than 30 years of age, Positive Likelihood Ratio (+LR) = Sensitivity / (1 - Specificity): 3.375 (CI95% 1.728–7.905) and Negative Likelihood Ratio (−LR) = (1 - Sensitivity) / Specificity 0.321 (CI95% 0.225–0.526). Chi Square - Mantel-Haenszel 22.194, $p = 0.000$

Diagnostic Biomarker Set			
A.			
Diagnostic Biomarker Set in women 30+ years of age	Diagnosis Positive (CIN2+)	Diagnosis Negative Cytology Negative)	Totals
Test Positive	119	21	140
Test Negative	60	35	95
Totals	179	56	235
B.			
Diagnostic biomarker set in woman <30 years of age	Dianosis positive (CIN2+)	Diagnosis negative (Cytology Negative)	Totals
Teat positive	51	6	57
Teat negative	17	21	38
Totals	68	27	95

been indicated previously as a cervical cancer biomarker. Those genes which are highly discriminative (considering both diagnostic and prognostic genes) have been implicated previously in cervical carcinogenesis.

Analysis of the results using PCA points to the underlying biological principles of the first two principal components (PC1, PC2) of PCA (percent of explained variance: 53.9 %), based on the distribution of the contribution vectors (Fig. 2, Panel b). The analysis revealed that all biomarker genes showed positive contribution to the first principal component (PC1) and that the grouping of biomarkers was markedly divided along the second principal component (PC2). As all biomarkers were selected on the basis of their over-expression in pre-cancer samples, PC1 was identified as a cellular response to HPV infection and/or proliferation. The separation of centroids was marked along the PC1 axis, which is compatible with the higher proliferative potential of the pre-cancer cases.

Table 3 Diagnostic performance values of the best gene panel differentiating CIN1 – carcinoma. A. Negative discriminatory gene panel: KRT78, MUC5AC and BPIFB (evaluated at threshold value of −1). Positive Likelihood Ratio (+LR) = Sensitivity / (1 - Specificity): 2.426 (CI95% 1.421–4.828) and Negative Likelihood Ratio (−LR) = (1 - Sensitivity) / Specificity 0.329 (CI95% 0.216–0.566). Chi Square - Mantel-Haenszel 18.117, $p = 0.001$. B. Positive discriminatory gene panel: CXCL13, TP63 and DSG3 (evaluated at threshold value of −1). Positive Likelihood Ratio (+LR) = Sensitivity / (1 - Specificity): 1.918 (CI95% 1.365–2.235) and Negative Likelihood Ratio (−LR) = (1 - Sensitivity) / Specificity 0.222 (CI95% 0.057–0.617). Chi Square - Mantel-Haenszel 13.746, $p = 0.000$

A.			
	Diagnosis Positive (CIN1)	Diagnosis Negative (CC)	Totals
Test Positive	66	8	74
Test Negative	19	17	36
Totals	85	25	110
B.			
	Diagnosis Positive (CC)	Diagnosis Negative (CIN1)	Totals
Test Positive	22	39	61
Test Negative	3	46	49
Totals	25	85	110

The separation of centroids of normal and carcinoma cases was greatest along the PC2 axis, this principal component was identified as the variable of oncogenicity/progression. These assignments were underlined by the marked separation of the negatively and positively contributing biomarker genes with biological functions compatible with this hypothesis. Regarding the PC2 axis, among the positive contributors ΔNp63 (TP63) isoforms frequently act to promote the oncogenic function [14], MUC5AC, LGALS7 and TP63 are associated with cell death, and DSG3 is linked to advanced CIN lesions [15]. On the contrary, EREG, IL1RN and KLK8 are linked to negative regulation of differentiation and response to wounding based on the querying of the DAVID functional annotation database [16], which predicts a role in physiological growth regulation. Moreover, our findings support the hypothesis that the member genes of the prognostic panels are over-represented in the positive contribution group, with the notable exception of KRT78, which is one of the strongest negative contributors. KRT78 contributes to the panel's discriminatory nature by identifying the least progressive lesions.

Desmoglein 3 (DSG3) is a structural protein involved in the formation of desmosomes, ie. cell-cell junctions between epithelial, myocardial and certain other cell types. DSG3 has

been identified as the auto-antigen of the autoimmune skin disease *pemphigus vulgaris*. Abnormal expressions of DSC3 (desmocollin 3), DSG3 and beta-catenin are found in the progression of oral carcinomas. DSC3 expression level might be related to the regulation of beta-catenin in lymph node metastasis and cell proliferation in oral squamous cell carcinomas [17]. There are data strongly supporting the view that DSG3 contributes to the regulation of epidermal differentiation and that over-expression of this protein indicates poor prognosis in lung cancers, and is associated with head and neck cancer. Its over-expressed state at messenger RNA level in cervical precancer and cancer was previously disputed [15], however recent immuno-histochemical findings have confirmed its over-expression at the protein level as well (unpublished results). In *pemphigus vulgaris* DSG3-mediated signaling pathways are activated with increased proliferation due to c-Myc accumulation [18, 19], along with DSG3-mediated activation of the PI3K/Akt pathway. The expression of PIK3AP1, one of the members of this latter pathway, was also a biomarker of cervical cancer in our study.

According to the clusterogram (Fig. 1), TP63 expression patterns were closely related to DSG3 patterns. This is underlined by recent studies in which TP63, the master regulator of epidermal development and differentiation, with the help of KLF4 (Kruppel-Like Factor 4) was capable of inducing DSG3 expression (among other keratinocyte specific genes) in human fibroblasts [20]. One of TP63's isoforms (DeltaNp63) is predominantly expressed in the basal cells of stratified epithelia (with inherent self-renewing capacity) and plays a fundamental role in the control of regenerative potential and epithelial integrity. TP63 is rarely mutated in human cancers, but is frequently over-expressed in squamous cell carcinomas (SCC). DeltaNp63alpha maintains the self-renewing capacity and consequently the proliferation of normal human keratinocytes and cervical cancer cells, partly through transcriptional repression of the Notch1 gene, therefore the frequently observed over-expression of DeltaNp63 may be pathologically significant [21]. Previously, TP63 was implicated in cervical cancer [22]. In the uterine cervix, expression of DeltaNp63 was found to be increased with the progression of CIN, and was positive in all SCCs, transitional cell carcinomas, and adenoid basal carcinoma, but negative in all adenocarcinomas [23].

CXCL13 is a B lymphocyte chemo-attractant, strongly expressed in the follicles of the spleen, lymph nodes and Peyer's patches. According to the clusterogram, it has distant relationship to the other biomarker gene expression patterns. It preferentially promotes the migration of B lymphocytes (compared to T cells and macrophages) expressing Burkitt's lymphoma receptor 1 (BLR-1). LtαβR (Lymphotoxin αβ receptor)-expressing mesenchymal cells that organize lymph node formation are orchestrated by chemokines including CCL19, CCL21 and CXCL13, and frequently initiate the formation of tertiary lymphoid structures in different tumor tissues [24]. Prostate-associated lymphoid aggregates, frequently below the epithelia and arranged in the B cell follicles express CXCL13, and the para-follicular T cell areas show CCL21 expression. Moreover, CXLC13 has been implicated in the bone metastasis of head and neck squamous cell carcinomas [25], neuroblastomas [26] and prostate cancer [27]. In our study, CXCL13 is linked to cervical cancer progression as it is a member of the CIN1/carcinoma differentiating prognostic biomarker gene panel. The association of CXCL13 with the advanced malignant phenotype is compatible with its role in the metastasis of other cancers and has been underlined by immunological studies that associate heavy intra-tumoral B cell infiltration [28] with cervical cancer.

PIK3AP1 has been identified as a tumor suppressor gene and has been reported to be inactivated by both promoter methylation and/or somatic mutation in head and neck cancers (HNCC) [29]. According to PCA, this gene is clearly a negative contributor along the oncogenicity axis (PC2) and is a member of the positive discriminatory gene panel of CIN1/carcinoma discrimination. In case of breast cancer, the PIK3AP1 gene region is a strong candidate for early onset breast cancer [30] and prostate cancer risk is associated with SNPs in PI3K genes including PIK3AP1 [31]. Our findings indicate close clustering of EREG and PIK3AP1. Inhibition of autocrine EREG action with cetuximab causes the inhibition also of cell growth in HNCCs [32]. This indicates that the function of PIK3AP1 may be linked to proliferation and cell growth.

KRT78 and other keratins are all part of a gene and linkage network that integrates barrier functions of the skin, and they have been identified as being part of the 'barrier–inflammation–cancer' network [33]. TP63 knock-outs show marked decrease in expression of KRT78 indicating its role in keratinocyte differentiation [34]. Increased TP63 in our samples was accompanied by increased KRT78 expression. In addition to this, the external noxa induced dermal wounding transcriptomic response also involves the increase of KRT78 [35]. MUC5AC is an anti-pathogen, secreted mucin, which has physiological function in the gut, lungs as well as the genital membranes. Its over-expression was seen in some carcinomas, e.g. in hidradenocarcinoma, an uncommon malignant intradermal tumor of the sweat gland [36].

BPIFB1 is highly expressed in nontumor nasopharyngeal epithelial tissues, but its expression is reduced in nasopharyngeal carcinoma (NPC), indicating that BPIFB1 may be associated with the tumorigenesis of NPC. Moreover, BPIFB1 also delays cell cycle progression from G1 to S phase and inhibits the expression of cyclin D1, cyclin-dependent kinase 4 (CDK4) and phosphorylated Rb, having a profound effect on tumor homoeostasis [37]. In our study, BPIFB1 over-expression positively contributed to carcinogenesis, which is a conflicting result compared to its anti-oncogenic function. However, as the HPV E7 function is to release E2Fs to

promote cell-cycle [38], limiting the availability of Rb can augment this effect and can be seen as an ineffective feedback mechanism to institute cell cycle arrest and paradoxically promote carcinogenesis. A very similar ineffective feedback loop has been described for p16INK4a and CDKs [39].

Taken together, the biological functions of the biomarker genes support their role in the identification of cervical pre-cancer and cancer. Accordingly, the supporting evidence in the literature shows that the panels are related to the differentiation, proliferation, metastasis and immune evasion of cervical cancer. This highlights their involvement in fundamental pathological processes and therefore their potential diagnostic value.

Our findings support our view according to which the selection of biomarker genes must be carried out under clinical circumstances. The involvement of CXCL13, which is related to the immune evasion of cervical cancer and GCs, is a clear indication of the success of this approach. The level of gene expression of potential biomarkers can only be validated properly in real clinical samples. In a routine cervical cytological sample (which is the only clinically relevant sample type) the diseased cells are under-represented compared to the normal cells and as a consequence the altered gene expression profile of the lesion could be masked by the gene expression of the background normal cells, therefore biomarkers must be highly over-expressed to combat this.

Screening is the first line public health measure to reduce the burden of cervical cancer. With cytology in the centre of the present protocol the number of unnecessary treatments has been assessed by several studies. According to Hopman at al. [40], 20 % of low-grade lesions were upgraded and 26 % of high-grade lesions were downgraded after review. In screening, the low PPV of a diagnostic test that directly precedes colposcopy not only has consequences on the colposcopy workload and costs, but also leads to unnecessary treatment. This consideration is independent of the type of first-level test, i.e. cytological examination or HPV testing. We have identified and clinically validated a signature biomarker gene panel for differentiation of hrHPV positive CIN2 or worse cases, as a triage test.

There are different risk-benefit considerations for women of different ages, as reflected in age-specific screening recommendations [41]. Our signature biomarker panel genes were evaluated in the light of the gynaecological screening recommendation that hrHPV testing is not recommend under age 30, as a co-test, and that its triage test value is also not established. We have detected insignificant differences between the age groups using PIK3AP1, TP63 and DSG3 genes in hrHPV populations comparing woman 30+ years of age and those <30 years.

Findings from our study indicated that some signature biomarkers panels (eg. CXCL13, TP63 and DSG3) can differentiate between CIN1 and carcinoma. This heralds the possibility of prognostic signature gene panels for cervical pre-cancer and cancer. Moreover, our finding that the expression of these genes increases according to the progression of the cervical lesions (unpublished results), and consequently the sensitivity of detection also increases, underlines the strong association of this biomarker gene-panel with the progression of cervical lesions and therefore warrants further investigation of its clinical utility.

Cervical cancer screening HPV triage tests, to stratify HPV positive cases, are the next necessary breakthrough in cervical cancer screening protocols, our aim was to establish new biomarkers in this field. Without effective triage tests, the cervical screening protocols face significant problems as the widespread and increasing acceptance of the primary HPV screening tests will decrease the PPV (positive predictive value) of cervical cancer screening to unacceptable level, which is further complicated by the declining HPV prevalence, due to worldwide vaccination efforts. Our biomarker panels have potential application in the traditional triage role (e.g. diagnostic panel) where triaging HPV+ women will increase the PPV value due to the increased specificity of overall screening when these markers are incorporated. However the prognostic panels herald a new potential application, which is an uncharted territory of the clinical management of the cervical cancer, as they might be useful tests as carcinogenic follow-up measures. This way HPV positive woman with proven progressive cervical pre-cancers lesions can be intercepted much more effectively than is currently possible.

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Compliance with Ethical Standards

Conflict of interest The corresponding author of this work is the inventor of a pending patent related to this work and Cellcall Ltd. owns this patent.

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