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Tissue-based Immunohistochemical Biomarker Expression in Malignant Glandular Lesions of the Uterine Cervix: A Systematic Review

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Introduction

Between 10–25% of carcinomas of the uterine cervix are adenocarcinomas [1]. The 2003 World Health Organization (WHO) classification listed 18 histotypes of primary, malignant glandular tumors whereas the 2014 update listed19 [1, 2]. Most histotypes are an endocervical type of mucinous adenocarcinoma, but rarer types such as minimal deviation adenocarcinoma-gastric type adenocarcinoma (MDA-GAS) and mesonephric carcinoma also occur. Endocervical adenocarcinoma in situ (AIS) is considered the precursor lesion of endocervical type, mucinous adenocarcinoma, whereas atypical lobular endocervical glandular hyperplasia (LEGH) is the proposed precursor of MDA-GAS.

High risk human papillomavirus (HPV) deoxyribonucleic acid (DNA) is detected in 94% of AIS, 85% of adenosquamous carcinomas and 76% of adenocarcinomas [3]. When stratified by histotype, HPV DNA is most commonly detected in endocervical adenocarcinoma, usual type (90%) and is progressively less common in serous (30%), clear cell (27%), and endometrioid carcinoma (13%). In contrast, atypical LEGH, MDA and the newly defined GAS histotype which is considered a poorly differentiated MDA variant are unrelated to the HPV [4–6]. When the HPV E7 protein competes with the transcription factor E2F for its pRB (Retinoblastoma) binding site, the subsequent loss of pRB function leads to p16 overexpression via an upregulated feedback loop [7]. Thus p16 overexpression has become a surrogate marker of HPV DNA positive cervical neoplasia and can be detected as strong diffuse nuclear and/or cytoplasmic staining using immunohistochemistry (IHC).

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There are many publications describing the IHC expression of p16 and various other biomarkers in malignant lesions of the uterine cervix. Based on the recommendations of the LAST (Lower Anogenital Tract Squamous Terminology) consensus meeting, p16 IHC is a sensitive and specific biomarker test in the diagnosis of HPV DNA positive cervical squamous intraepithelial lesions (SIL) and squamous cell carcinomas (SCC) [8]. The role of HPV in glandular malignancies suggests p16 IHC may also be a useful diagnostic biomarker. In our recently published systematic review and meta-analysis (SRMA) of the IHC biomarker literature on glandular malignancies of the uterine cervix, we determined p16 was a sensitive and specific biomarker in distinguishing cases of glandular malignancy from such negative controls as normal glandular epithelium and benign glandular lesions of the cervix (case-control analysis) [9]. However, whether IHC biomarker expression can distinguish the different glandular histotypes from each other has not been systematically analysed. The goal of this additional study of the SRMA data was to conduct a case-comparator study of IHC biomarker expression amongst the various glandular histotypes so as to identify differences between them that could have diagnostic utility.

Methods

Details of the SRMA search strategy for articles of tissue-based, IHC biomarker expression used in the case-control analysis, and the criteria and processes used in triaging the articles for final selection were previously published [9]. Briefly, abstracts of all potential reports were screened for study eligibility and data on 22 attributes which included IHC biomarker name, expression scoring details and positive-negative cut offs, case type and sample size, comparator type and sample size, and number of positive and negative test results were extracted and entered into a customized electronic spreadsheet. The final selection of articles thereafter was based on an evaluation for quality using Quality Assessment of Diagnostic Accuracy version 2 (QUADAS-2) [10]. A PICOT (Population, Index test, Comparator, Outcomes, Time interval) framework was applied [11]. The Index test was IHC biomarker expression in tissue samples, and the Time interval for the first literature search spanned January 1, 1975 to December 31, 2013: 2 separately conducted update searches were concluded June 30, 2015. The Population (cases) consisted of AIS, MDA-GAS, and all other primary invasive adenocarcinomas of the uterine cervix classified per WHO 2003 [2]. The Comparator consisted of atypical LEGH, MDA-GAS, and all other primary invasive adenocarcinomas of the cervix classified per WHO 2003. The main Outcome was the prevalence of positive biomarker IHC expression.

Various terminologies were used in the articles to classify the adenocarcinomas. To enable case-comparisons, adenocarcinoma cases were grouped as 1) mucinous adenocarcinoma, 2) endometrioid adenocarcinoma, 3) adenosquamous carcinoma, 4) serous and clear cell carcinoma, 5) MDA-GAS, and 6) mesonephric carcinoma based on morphological similarities and/or etiological associations. Mucinous adenocarcinoma cases included tumors classified as mucinous adenocarcinoma, mucinous adenocarcinoma NOS (Not Otherwise Specified), endocervical adenocarcinoma, villoglandular adenocarcinoma, mild, moderately and poorly differentiated adenocarcinoma, intestinal adenocarcinoma, signet ring carcinoma and superficially invasive adenocarcinoma. Adenosquamous carcinoma cases also included any tumors classified as glassy cell carcinoma, and MDA-GAS included minimal deviation,

endometrioid adenocarcinoma. Adenocarcinoma comparators were grouped in the same way. This generated 5 comparator groups for each of the 6 case groups (30 Adenocarcinoma case-comparators). AIS cases were compared to the 6 adenocarcinoma groups and in addition were compared to atypical LEGH (7 AIS case-comparators). Results of individual biomarker positivity in samples across studies were pooled to develop a combined estimate for each biomarker in the cases and comparators. To examine if patterns of biomarker positivity differed between cases and comparators an analytical framework of unsupervised hierarchical clustering, with complete linkage and a Euclidian distance metric was used (Cluster 3.0 open access software-ware. http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Biomarker positivity estimates were simultaneously clustered across the cases and comparator groups, and the clustering was visualized via heatmaps and dendrograms (Java TreeView open access software. http://jtreeview.sourceforge.net). The heatmaps displayed biomarker positivity of 100% as red, 0% as black and percentages in between as shades of these 2 colors. Useful biomarkers were identified by relative differences in color between the cases and comparators. They were also identified in the dendrograms by the relative distribution differences in the distance ("Euclidean distance") reflecting the arrangement of the biomarkers produced by the hierarchical clustering.

Results

There were 902 records (articles) identified in the first search and 154 were selected for a full review and data extraction. Details of the additional records and inclusions and exclusions are shown in Figure 1. The most frequent reason for article exclusion was the absence of a defined IHC positive-negative cut off. The final dataset consisted of 52 articles with results for 56 unique IHC biomarkers (Glossary) [12–63]. Biomarkers with a 50% or more difference in positive expression were identified and considered to be diagnostically useful.

AIS case-comparators

There was data on the positive expression of 1 or more of 20 biomarkers in AIS cases versus the 7 comparator groups [13–31, 33, 35, 39, 40, 43, 44, 47, 48, 52–55, 63]. p16, HIK1083 and CD10 expression were the most frequently compared and each was compared in 6 of the 7 comparators. Two biomarkers (Epithelial Specific Antigen and pRB) were compared once and only in comparison to mucinous adenocarcinoma. Biomarker positivity was variable amongst the case-comparators (Figure 2). In the comparison of 7 biomarkers with atypical LEGH, the positivity difference ranged from 8% to 59% and only HIK1083 had a difference of 50% or more (Figure 2a). Of the 19 biomarkers evaluated in the comparison to mucinous adenocarcinoma, only Alpha SMA expression in the peri-lesional stromal cells showed a positivity difference of 50% or more (Figure 2b). None of the 8 biomarkers tested in comparison to endometrioid adenocarcinoma had a positivity difference of 50% or more (Figure 2c) but PAX 8 and VIL1 amongst the 8 biomarkers compared to adenosquamous carcinoma did (Figure 2d). The comparison of 4 biomarkers to serous-clear cell carcinoma showed CEA and p53 had a 50% or more difference in positivity (Figure 2e). In the comparison with MDA-GAS, alpha SMA, HIK1083, p16 and p53 of the 13 biomarkers compared showed a 50% or more difference in positivity and the widest was with alpha SMA (Figure 2f). Only CD10 expression compared to mesonephric carcinoma had a 50% or

more difference in positivity (0.08 vs. 0.67) [26, 29, 48]. Alpha SMA expression in comparison to mucinous and MDA-GAS was the only biomarker with a positivity difference of 100%.

Adenocarcinoma case-comparators

There was data on the positive expression of 1 or more of 36 biomarkers in adenocarcinoma cases versus the 5 comparators. p16, ER, PR, HIK1083, and CD10 were the most frequently compared and each of these 5 biomarkers was compared in at least 4 of the 5 comparators. CD10 and Calretinin were the only 2 biomarkers evaluated in the mesonephric case-comparators. Biomarker positivity was variable amongst the 30 case-comparators and differences of 50% or more did occur. No biomarker showed a positivity difference of 100% amongst any of the case-comparators.

There was data on the positive expression of 1 or more of 36 biomarkers in mucinous adenocarcinoma cases versus the 5 comparator groups [13–19, 21–23, 25–35, 38–44, 46–49, 51–55, 58, 61–63]. A total of 19 biomarkers were compared once and only to 1 of 3 comparators: adenosquamous (MCM7, p63, PTEN, VIL1), endometrioid (CTK20, CTK7, telomerase, ubiquitin) and MDA-GAS (alpha SMA, CA-125, CA-IX, CDX2, Claudin18, KI67, PAX2, pCEA, PNCA, SMA, TTF1). There was a 37-0% difference in positivity amongst the 17 biomarkers evaluated in the comparison to endometrioid adenocarcinoma (Figure 3a). Amongst the 13 biomarkers compared to adenosquamous carcinoma (Figure 3b), p63 had a positivity difference of 94%. Out of 25 biomarkers evaluated in the comparison to MDA-GAS, Claudin18, HIK1083, and p16 showed a difference of 50% or more and the widest was with Claudin18 (Figure 3c). The comparison of 8 biomarkers to serous-clear cell carcinoma showed CEA and p53 had a positivity difference of 50% or more (Figure 3d) as did CD10 (0.11 vs. 0.67) [29, 48] and Calretinin (0.10 vs. 0.67) [48] in comparison to mesonephric carcinoma.

There was data on the positive expressions of 1 or more of 17 biomarkers in endometrioid adenocarcinoma cases versus the 5 comparators [15–17, 22, 23, 25, 26, 28, 29, 30, 31, 33–35, 37–39, 40, 42–44, 47–49, 52–55, 62, 63]. A total of 5 biomarkers (ubiquitin, telomerase, CTK20, CTK7, and EMA) were compared once and only in comparison to mucinous adenocarcinoma. Amongst 9 biomarkers compared to adenosquamous carcinoma, PR was the only one with a 50% plus positivity difference (Figure 4a). Chromogranin, HIK1083, MUC6, p16, PR, and Vimentin out of the 10 biomarkers evaluated in the comparison to MDA-GAS showed a positivity difference of 50% or more and the widest difference was with HIK1083 (Figure 4b). The comparison of 6 biomarkers to serous-clear cell carcinoma showed only CEA and PR had a 50% or more difference in positivity (Figure 4c), and the comparison of CD10 to mesonephric carcinoma had a positivity difference of only 34% (0.33 vs. 0.67) [29, 48].

There was data on the positive expression of 1 or more of 11 biomarkers in adenosquamous carcinoma cases versus the 5 comparators [15, 16, 22, 25, 28, 29, 38, 39, 40, 43, 44, 47, 48, 52, 53, 55, 62, 63]. PTEN and VILI1 were compared once and only in comparison to mucinous adenocarcinoma. Positivity differences amongst CD10 (0.00 vs. 0.11) [29, 48], Chromogranin (0.06 vs 0.26) [29], ER (1.00 vs 0.77) [38, 43, 47, 52, 63], HIK1083 (0.00 vs. 0.09) [29, 44, 47, 52], MUC2 (0.00 vs 0.28) [29], MUC6

(0.05 vs 0.26) [29, 44, 47], p16 (0.94 vs 0.89) [16, 25, 28, 29, 40, 44, 47, 52, 53], PAX8 (0.42 vs 0.65) [39, 55, 62], PR (1.00 vs. 0.75) [38, 43, 52, 63], PTEN (0.91 vs. 0.91) [15], and VIL1 (0.20 vs. 0.52) [22] were all less than 50% when compared to mucinous adenocarcinoma (heatmap and dendrogram not shown). Of the 9 biomarkers evaluated in the comparison to MDA-GAS, chromogranin, HIK1083, and p16 showed a difference of 50% or more and the widest difference was with HIK1083 (Figure 5). Differences were less than 50% in the comparison of ER (1.00 vs. 1.00) [38, 52], HIK1083 (0.00 vs. 0.00) [29, 5]), p16 (0.94 vs. 0.98) [16, 28, 29, 52, 53], and PR (1.00 vs 1.00) [38, 52] to serous-clear carcinoma. Only CD10 was compared to mesonephric carcinoma and it had a difference of more than 50% (0.00 vs. 0.67 [29, 48]).

There was data on the positive expression of 1 or more of 8 biomarkers in serous-clear cell carcinoma cases versus 4 of the 5 comparator groups [16, 17, 25, 28, 29, 30, 33, 34, 35, 38, 40, 43, 44, 47, 52, 54, 63]. There was no data comparing the case histotypes to mesonephric carcinoma. Out of the 7 biomarkers evaluated in the comparison to MDA-GAS, CEA, HIK1083, and p16 had a positivity difference of 50% plus and the widest difference was with HIK1083 (Figure 6).

Results for the remaining case-comparator analyses were similar to those already obtained when the comparator was the case and case was the comparator except that the positivity results were reversed. Biomarkers with a 50% plus positivity difference in these remaining analyses were thus: PR [38, 43] for adenosquamous versus endometrioid, CEA [17, 33, 35, 43, 52, 54, 63] and p53 [30, 52, 63] for serous-clear cell versus mucinous, CEA [17, 33, 43, 52] and PR [38, 43, 52] for serous-clear cell versus endometrioid, Claudin18 [47], HIK1083 [29, 44, 47,52] and p16 [16, 25, 28, 29, 40, 44, 47, 52, 53] for MDA-GAS versus mucinous, chromogranin [29], HIK1083 [29, 44, 47, 52], MUC6 [29, 44, 47], p16 [25, 29, 40, 44, 47], PR [38, 43, 52, 63], and Vimentin [27, 33, 43] for MDA-GAS versus endometrioid, chromogranin [29], HIK1083 [29, 44, 47, 52] and p16 [29, 40, 44, 47, 52, 53] for MDA-GAS versus adenosquamous, CEA [52], HIK1083 [29, 44, 47, 52], and p16 [16, 25, 29, 40, 44, 47, 52] for MDA-GAS versus serous-clear cell, Calretinin [48] and CD10 [29, 48] for MDA-GAS versus mesonephric, Calretinin [48] and CD10 [29, 48] for mesonephric versus mucinous, CD10 [29, 48] for mesonephric versus adenosquamous, and Calretinin [48] and CD10 [29, 48] for mesonephric versus MDA-GAS.

Discussion

The systematic review showed tissue based, IHC biomarker expression to discriminate malignant glandular histotypes of the uterine cervix from each other needs further study. Out of 56 biomarkers tested and detailed in 52 articles, 15 had a positivity difference of 50% or more and could have diagnostic utility in the discrimination of AIS from invasive adenocarcinoma, and in discriminating between some of the invasive adenocarcinoma histotypes (Table 1). Amongst 6 (86%) of the AIS case-comparators (exempted case-comparator=AIS versus endometrioid adenocarcinoma), 1 or more of 8 biomarkers (HIK1083, alpha SMA, PAX8, VIL1, CEA, p53, p16 and CD10) could be useful (Table 1a). Amongst 21 (70%) Adenocarcinoma case-comparators, 1 or more of 12 biomarkers (CEA, p53, Claudin18, HIK1083, p16, Calretinin, CD10, PR, Chromogranin, MUC6, Vimentin and p63) could be useful. The exemptions were comparisons of mucinous to endometrioid,

endometrioid to mucinous and mesonephric, adenosquamous to mucinous and serous-clear cell, serous-clear cell to adenosquamous, and mesonephric to endometrioid and serous-clear cell, and there was no data on the comparison of serous-clear cell to mesonephric carcinoma (Table 1b). Only alpha SMA expression had a positivity difference of 100% and this occurred when AIS was compared to mucinous adenocarcinoma and to MDA-GAS.

This is the first systematic review of the published literature on tissue based, IHC biomarker performance in the discrimination of the various malignant glandular histotypes of the uterine cervix from each other. The project is an extension of our previously published SRMA on the sensitivity and specificity of tissue-based, IHC biomarker expression in the diagnosis of malignant glandular lesions in comparison to normal cervix and benign glandular lesions [9]. In the current review of 52 included articles [12–63], biomarker expression amongst the histotypes was analysed by comparing the percentage of positive expression in malignant cases to malignant comparators. Diagnostic biomarkers with a 50% or more difference in expression were interpreted as potentially useful. The study was designed to analyse 37 case-comparator scenarios. Some case-comparators, e.g., AIS versus atypical LEGH would not need IHC to discriminate between them because the morphology of each lesion is so distinct. However we decided to investigate those scenarios as well so as to generate a comprehensive list of all possible case-comparators that the practicing pathologist would consider when interpreting the pathology of these lesions. To the best of our knowledge, this is also the first time an analytical framework of unsupervised hierarchical clustering with visualization via heatmaps and dendrograms has been used to compare biomarker expression in histotypes. We chose this methodology as it is more appropriate for the analysis of categorical data. Since the open access software is freely available and relatively simple to use, this approach could become the standard for future case-comparator studies of IHC biomarker expression.

HPV DNA is present in 94% of AIS, 85% of adenosquamous carcinomas and 76% of cervical adenocarcinomas [3]. Recent studies of MDA-GAS have confirmed this histotype is unrelated to HPV and instead appears to have origin in metaplastic lesions (e.g., atypical LEGH) with a gastric phenotype and molecular profile [3–6]. Mesonephric carcinoma originates from mesonephric duct remnants which are of Wolffian duct origin and is unrelated to the HPV and gastric metaplasia [1, 23]. Over-expression of p16 as a surrogate marker of HPV DNA occurs in glandular malignancies caused by the HPV and is absent or expressed in low levels in histotypes that are not associated [7]. The systematic review supports stratification of cervical adenocarcinomas into HPV positive and negative. Overexpression of p16 occurred in the comparisons of AIS, mucinous adenocarcinoma, endometrioid adenocarcinoma, adenosquamous carcinoma, and serous-clear cell carcinoma to MDA-GAS, and in the comparison of AIS to atypical LEGH. In contrast, the gastric marker HIK1083 was overexpressed when MDA-GAS was compared to mucinous, endometrioid, adenosquamous, and serous-clear cell carcinoma. The immuno-profile of mesonephric carcinoma cases and comparators was understudied, but the limited data supported its inclusion in the HPV negative category. Calretinin and CD10 expression was increased and this profile differed from those of the HPV positive and MDA-GAS adenocarcinomas.

Differences in the positive expression of p16, HIK1083, CD10 and Calretinin amongst HPV positive and negative adenocarcinomas never reached 100%, however. This may be due to variability amongst studies in the accuracy of histotyping and in the biomarker clones, scoring, and positive and negative cut offs used. The mucinous adenocarcinoma category of this study included a number of histotypes and thus may not be a homogenous group. For example, endometrioid adenocarcinoma is difficult to distinguish from mucin poor, mucinous adenocarcinoma [1]. Its inclusion in the mucinous category would therefore impact the positivity of biomarker expression in both carcinoma groups. Much of the variability however is more likely related to the biomarkers and the evaluation of the expression. For example, although not all of the AIS case-comparator studies provided full details, there were at least 4 different p16 clones, 5 different methods of evaluating expression, and 6 different positive-negative cut-off definitions [13, 14, 16, 19, 21, 25, 27–29]. The same was true for the adenocarcinoma case studies [13, 14, 16, 19, 21, 25, 27–29, 40, 41, 44, 46, 47, 51–53, 58, 61]. Thus diagnostic application of these biomarkers in the different case-comparator scenarios will be limited until further studies are conducted which control for misclassification of histotypes and use standardized biomarker scoring and cut offs which are consistently applied and validated.

Alpha SMA was the only biomarker that could distinguish in situ from invasive adenocarcinoma and it was also the only biomarker with a 100% difference in positivity in any of the case-comparator scenarios. It was seen in the distinction of AIS from mucinous adenocarcinoma and from MDA-GAS. Expression occurred in the stromal cells surrounding the invasive carcinoma. Thus evaluation of this biomarker might be very useful in determining whether AIS shows early stromal invasion and be correctly diagnosed as an invasive adenocarcinoma. This data however is from 1 study and has not been recapitulated [31]. Very few biomarkers emerged as useful in the distinction between the HPV positive invasive histotypes. p63 which is a keratin marker was overexpressed in adenosquamous carcinoma compared to mucinous adenocarcinoma, and p53 was overexpressed in serous-clear cell carcinoma compared to mucinous adenocarcinoma. Lower or higher expression of PR had some potential as a marker of endometrioid adenocarcinoma when compared to adenosquamous and serous-clear cell carcinoma respectively. Diagnostically useful biomarkers were not identified for 9 cases-comparators (AIS versus endometrioid, mucinous versus endometrioid, endometrioid versus mucinous and mesonephric, adenosquamous versus mucinous and serous-clear cell, serous-clear cell versus adenosquamous, and mesonephric versus endometrioid and serous-clear cell) although several biomarkers were tested, and there was no data comparing serous-clear cell to mesonephric carcinoma. Some of these results however, came from small descriptive studies or discovery research which were underpowered to show differences in expression. Thus further study of at least alpha SMA, p63, p53 and PR expression in certain case-comparator scenarios is needed as is the identification and study of new biomarkers such as Napsin A which is overexpressed in clear cell carcinoma of the ovary and endometrium but has not been investigated cervical tumors [64].

A systematic review as a timed publication is very challenging due to ever changing landscape of published new information. Twice the literature was updated for this review and even with this degree of diligence, literature from the date of the second update is

absent. Therefore we scanned the literature published in that 18 month period for information on any novel biomarkers and/or additional case-comparator analyses. We identified 5 publications that provided some new information and would have met our inclusion criteria [65–69]. For example, GATA 3 expression may be a marker of mesonephric lesions since it was positive in all mesonephric remnants and hyperplasias and nearly all mesonephric carcinomas tested, and infrequent or absent in endocervical adenocarcinomas, usual type and GAS [65–67]. The HPV viral protein E7 was not a discriminant in the comparison of AIS to cervical adenocarcinomas, but may be an additional discriminant of HPV positive and negative adenocarcinomas in situations where the HPV negative lesions may show a high frequency of p16 positivity [68, 69].

Ancillary diagnostic IHC generally involves the use of a panel of biomarkers rather than a single biomarker. Increasingly in the practice of pathology, it is becoming more common to base the interpretation of multiple biomarker results on an algorithm of sequential biomarker testing [70]. This approach improves the diagnostic performance of IHC. Thus the next steps of any study investigating the performance of tissue-based-IHC in the distinction of the different glandular histotypes from each other would be to test multiple adenocarcinoma examples that include all histotypes and all case-comparators with a panel of biomarkers from at least the 15 identified in this systematic review and with the possible addition of GATA 3 and HPV E7, to standardize and validate the IHC testing and scoring, and when appropriate use regression analysis to develop algorithms of sequential biomarker testing.

Glossary of 56 Biomarker Acronyms and/or Names

Alpha-SMA	Alpha smooth muscle actin
	Beclin-1
	CA125
CA-IX	Carbonic anhydrase
	Calretinin
	CD10
	CD44s
	CD44v3
	CD56
	CDX2
CEA	Carcinoembryonic antigen
	Chromogranin
	Claudin 18
	CK20
	CK7

	D2–40
	E Cadherin
EMA	Epithelial membrane antigen
	Epithelial specific antigen
ER	Estrogen receptor
	GATA3
	hENT1
	HIK1083
HNF1beta	Hepatocyte nuclear factor 1 beta
hPankoMab	humanized PankoMab (directed against a tumor related MUC1 epitope)
	Keratan sulfate
	Ki67
	L1 Capsid
	LC3B
MCM7	Minichromosome maintenance complex component 7
MMP-2	Matrix metalloproteinase 2
MUC2	Mucin 2
MUC6	Mucin 6
	MUC5AC
	p16
	p16+/Ki67+ dual stain
	P40
	p53
	p63
PAX2	Paired box gene 2
PAX8	Paired box gene 8
pCEA	Polyclonal carcinoembryonic antigen
PNCA	Proliferating cell nuclear antigen
PR	Progesterone receptor
pRB	Retinoblastoma protein

	ProExC
PTEN	Phosphatase and tensin homolog
	SMA
SOD2	Superoxide dismutase 2
SP17	Sperm protein 17
	Synaptophysin
	Telomerase
	TTF1
	Ubiquitin
VIL1	Villin 1
	Vimentin

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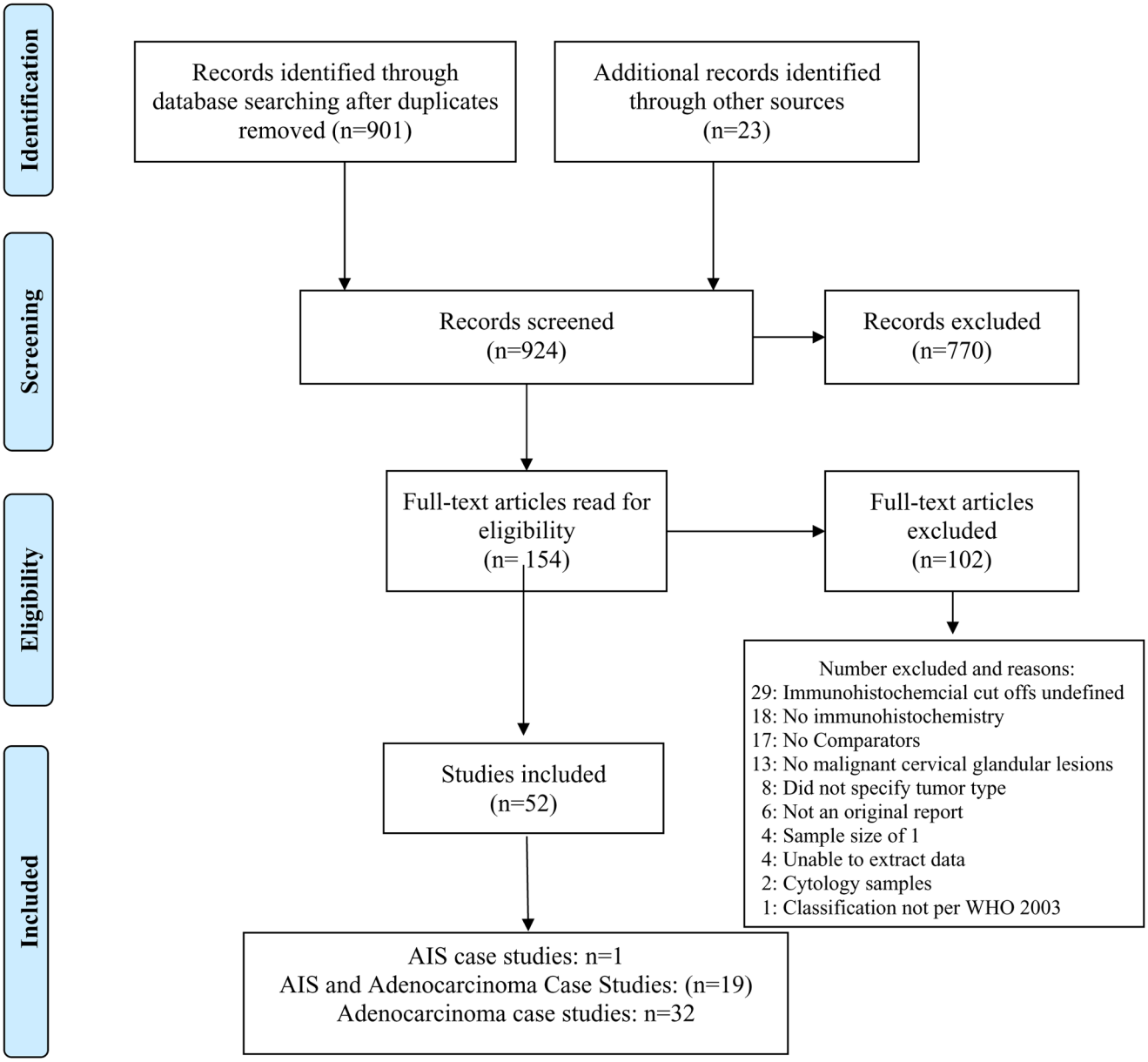

Figure 1.
Records and studies included and excluded in the systematic review

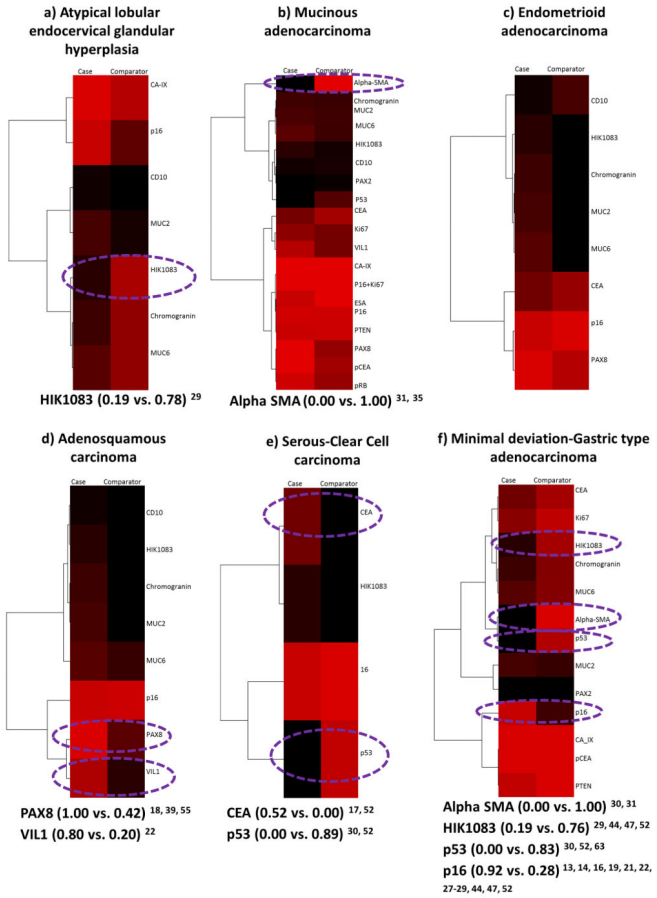

Figure 2.

Adenocarcinoma in situ case-comparators: heatmaps and dendrograms. Biomarker positivity of 100% is red, 0% is black and percentages in between are shades of these 2 colors. Biomarkers with a positivity difference of 50% or more are circled in purple. a) Atypical LEGH comparator. b) Mucinous adenocarcinoma comparator. c) Endometrioid adenocarcinoma comparator. d) Adenosquamous carcinoma comparator. e) Serous-clear cell carcinoma comparator. f) Minimal deviation/gastric type adenocarcinoma comparator.

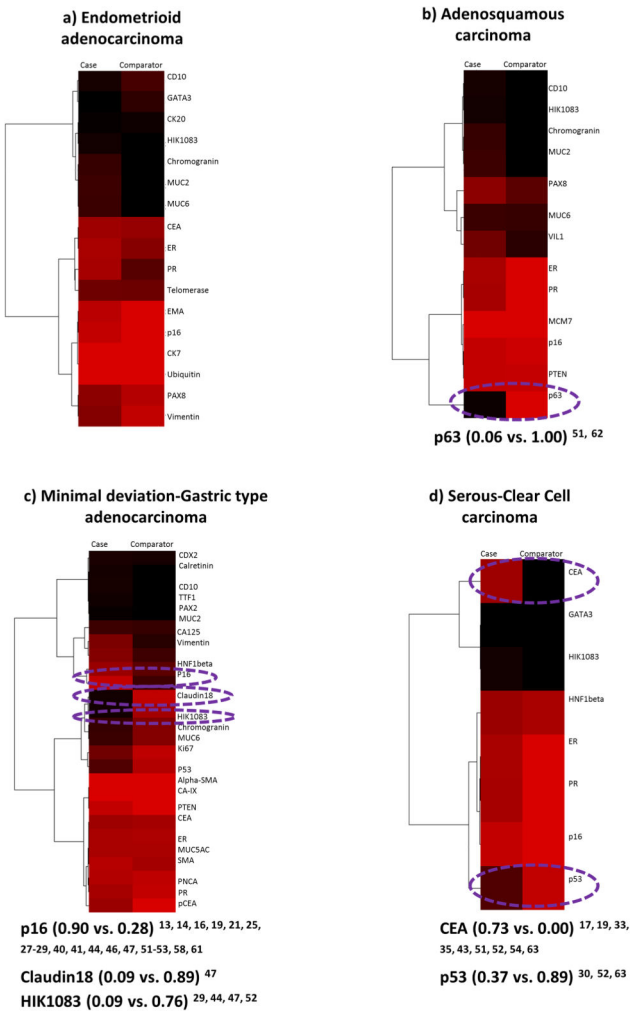

Figure 3.

Mucinous adenocarcinoma case comparators: heatmaps and dendrograms. Biomarker positivity of 100% is red, 0% is black and percentages in between are shades of these 2 colors. Biomarkers with a positivity difference of 50% or more are circled in purple. a) Endometrioid adenocarcinoma comparator. b) Adenosquamous carcinoma comparator. c) Minimal deviation/gastric type adenocarcinoma comparator. d) Serous-clear cell carcinoma comparator.

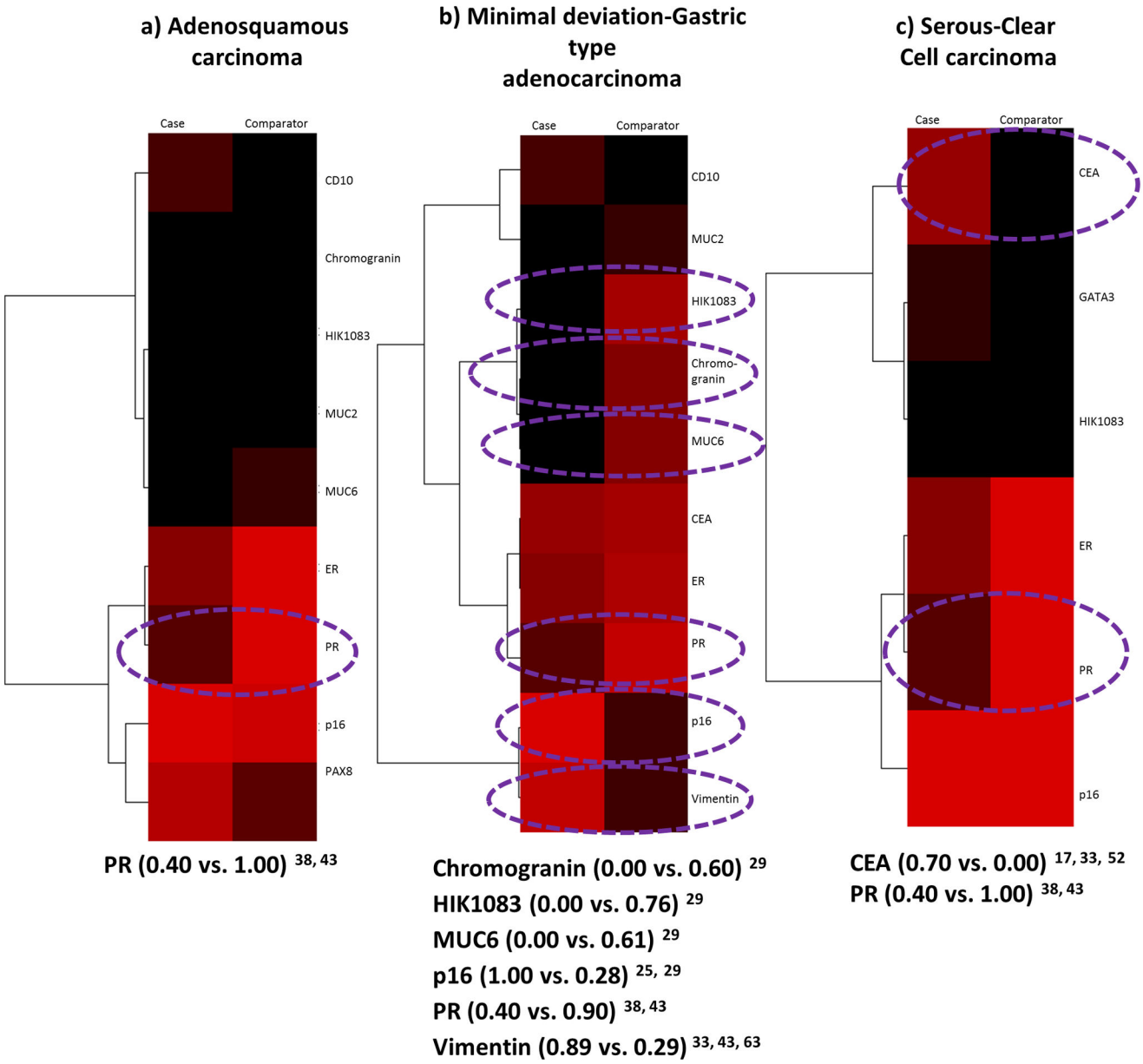

Figure 4.

Endometrioid adenocarcinoma case-comparators: heatmaps and dendrograms. Biomarker positivity of 100% is red, 0% is black and percentages in between are shades of these 2 colors. Biomarkers with a positivity difference of 50% or more are circled in purple. a) Adenosquamous carcinoma comparators. b) Minimal deviation/gastric type adenocarcinoma comparator. c) Serous-clear cell carcinoma comparator.

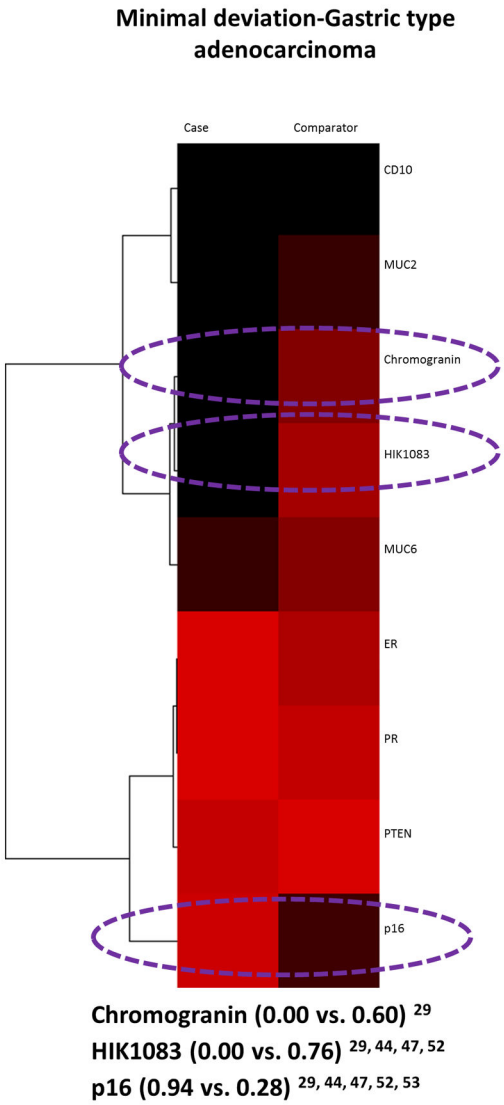

Figure 5.

Adenosquamous carcinoma case versus Minimal deviation/gastric type adenocarcinoma comparator: heatmap and dendrogram. Biomarker positivity of 100% is red, 0% is black and percentages in between are shades of these 2 colors. Biomarkers with a positivity difference of 50% or more are circled in purple.

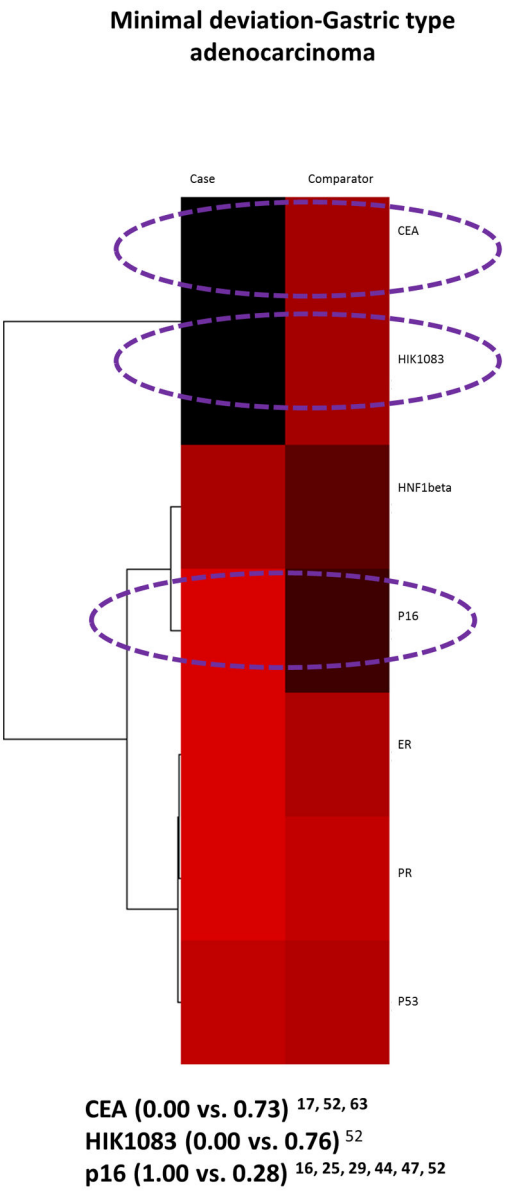

Figure 6.

Serous-Clear cell carcinoma case versus Minimal deviation adenocarcinoma/Gastric type adenocarcinoma comparator: heatmap and dendrogram. Biomarker positivity of 100% is red, 0% is black and percentages in between are shades of these 2 colors. Biomarkers with a positivity difference of 50% or more are circled in purple.

Table 1

Biomarkers with a 50% or more difference in positivity: a) AIS case-comparators and b) Adenocarcinoma case-comparators

Table 1a Casesversus comparators	Atypical LEGH	Mucinous adeno-carcinoma	Endometrioid adeno-carcinoma	Adeno-squamous carcinoma	Serous-clear cell carcinoma	MDA-GAS	Mesonephric carcinoma
AIS	HIK1083	Alpha-SMA*		PAX8VIL1	CEAP53	Alpha-SMA* HIK1083p16p53	CD10

Table 1b Cases versus comparators	Mucinous adeno-carcinoma	Endometrioid adeno-carcinoma	Adeno-squamous carcinoma	Serous-clear cell carcinoma	MDA-GAS	Mesonephric carcinoma
Mucinous adenocarcinoma			p63	CEA p53	Claudin 18 HIK1083 p16	Calretinin CD10
Endometrioid adenocarcinoma			PR	CEA PR	Chromo-granin HIK1083 MUC6 p16 PR Vimentin	
Adenosquamous carcinoma		PR			Chromo-granin HIK1083 p16	CD10
Serous-clear cell carcinoma	CEA p53	CEA PR			CEA HIK1083 PR	No data
MDA/GAS	Claudin18 HIK1083 p16	Chromo-granin HIK1083 MUC6 p16 PR Vimentin	Chromo-granin HIK1083 p16	CEA HIK1083 p16		Calretinin CD10
Mesonephric carcinoma	Calretinin CD10		CD10		Calretinin CD10	

Grey: none with greater than 50% difference in positivity

*Positivity difference=100%