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Hypoxic-inducible Factor (HIF1α) and Aldolase C Protein Immunoexpression in Endometrial Cancer

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Hypoxic-inducible Factor (HIF1α) and Aldolase C Protein Immunoexpression in Endometrial Cancer

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Abstract:

Objectives: Hypoxia-inducible factor (HIF1α) plays an integral role in response to hypoxia, controlling dozens of target genes including aldolaseC (ALDC), an important enzyme in the glycolytic pathway. It also induces angiogenesis, allowing survival and proliferation of cancer cells. Thus, inhibition of HIF1α may be an attractive therapeutic option. The aims of our study were to (1) evaluate the expressions of HIF1α and ALDC in patients with endometrial cancer (EC) and define their association with disease outcome and (2) determine the existence of an association between HIF1α and ALDC proteins. **Design:** We performed immunohistochemistry using antibodies to ALDC and HIF1α on paraffin-embedded tissue from 279 patients. The association of ALDC and HIF1α immunoexpression was evaluated, as well as the association of their expressions with the following clinical parameters; age, histologic subtype, myometrial depth of invasion, lymphovascular invasion, FIGO grade, lymph nodes status, and disease status. **Results:** ALDC and HIF1α were overexpressed in the vast majority of EC cases (78% and 76% respectively). There was a strong positive association between HIF1α and ALDC (p=0.0017). There was a significant association between ALDC and depth of myometrial invasion (p=0.0438), and between HIF1α and tumor grade (p= 0.0231) and tumor subtype (p=0.018). However, there was no association between either ALDC nor HIF1α and disease status. **Conclusions:** ALDC and HIF1α play an important role in endometrial carcinogenesis. Their expression by the majority of EC makes inhibition of HIF1α a very attractive therapeutic option for treating patients with EC.

Key words: HIF1α; AldolaseC; Immunohistochemistry; Endometrial cancer; Disease outcome.

Subject headings: HIF1α and aldolaseC in endometrial cancer

Article Summary:

1- Article focus:

- HIF1 α and aldolase C expressions in patients with endometrial cancer
- HIF1 α and aldolase C interaction in vivo in endometrial cancer.
- HI1 α and aldolaseC value in predicting disease outcome in patients with endometrial cancer.

2- Key messages:

- HIF1 α and aldolase C are frequently expressed in endometrial cancer.
- There is strong association between HIF1 α and aldolase C in endometrial cancer and therefore they could play an important role in its pathogenesis.
- HIF1 α and aldolase C are associated with poor prognostic factors.
- HIF1 α and aldolase C are not independent predictive biomarkers of poor outcome.

3- Strengths:

- It is a large study of 279 patients.
- It is one of the few in the literature.

- It is the first to evaluate the association of HIF1 α and aldolase C in vivo in endometrial cancer.

Limitations:

- The study did not have a large numbers of type II cancers (serous and clear cell carcinomas).
- The study did not contain too many cases of late stage tumors (stage III and IV).

Study approval:

This study was approved by the IRB committee and was conducted with maintenance of respect to privacy of all patients throughout.

Funding statement:

We, the authors, declare that there was no funding for this study.

Competing interests:

We, the authors, declare that there is no competing interest with this study.

Contribution statement:

PMF: designed and wrote the study

DW, SL: performed the statistical analysis

DS, HG, TM: reviewed the patients charts

DS, FO: helped in reviewing the manuscript.

SL (Shashikant Lele), TP: mentored and were consulted in designing, conducting and writing the study

Introduction:

Endometrial cancer (EC) is the most common gynecologic malignancy in developed countries. There are approximately 42,000 cases diagnosed annually in the United States, resulting in almost 8,000 deaths, [1]. EC has been classified into two types based on morphology, pathogenesis, behavior and treatment: type I (endometrioid and mucinous carcinomas), and type II (serous and clear cell carcinomas). Type I is usually low-grade and low stage at initial presentation. Type II is usually high grade and advanced stage at initial presentation. The most reliable prognostic factors in predicting disease outcome in EC are tumor grade, tumor stage, tumor subtype, depth of myometrial invasion, and lymph node involvement, [2-4].

One of the most prominent metabolic alterations in cancer cells is an increase in aerobic glycolysis, known as the Warburg effect after its discovery by Otto Warburg in 1920, [5]. This increase in glycolysis, due to a shift in glucose metabolism from oxidative phosphorylation into the aerobic glycolysis pathway, provides the tumor with metabolic and survival advantages, [6-8]. Aldolase, a critical enzyme in the glycolytic pathway, catalyses the reversible conversion of fructose-1,6-biphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Aldolase has three distinct isoenzymes, A, B, and C, which are similar in sequence with 78% identity between A and C and 68% identity between B and C, [9,10]. Originally identified in brain tissue, aldolaseC (ALDC) has been seen to be overexpressed in carcinomas of the lung, kidney, cervix and endometrium, [11-13].

The Hypoxic-inducible factor (HF1) gene codes for two subunits, α and β, and is usually activated by hypoxic conditions, a microenvironment that commonly accompanies cancerous tumors. When activated, HIF1 can interact with enzymes and other transcription factors in order to control vascularization and tissue growth. HIF1α was recently identified as a potent regulator of ALDC, another mechanism by which it may promote carcinogenesis, [14]. Thus, attempts to target the HIF1α pathway in hopes of suppressing cancer cell proliferation and progression are underway. In the gynecologic tract, HIF1α expression increases as the endometrium undergoes changes from normal to premalignant to endometrioid adenocarcinoma. This is paralleled by increased angiogenesis in the endometrium suggesting that HIF1α might be a key regulator in endometrial carcinogenesis, [15].

Though the interaction between HIF1α and ALDC has been seen in vivo, their interaction in human samples and in endometrial carcinoma has not yet been described. Therefore, the aims of this study are (1) to evaluate the expression of HIF1α and ALDC proteins in patients with endometrial cancer and to find an association between these two proteins in this patient sample and 2- to determine whether either of these two proteins independently or any combination of their expressions might have an impact on disease outcome.

Materials and Methods:

Patient population: After obtaining IRB approval, the pathology archives were searched for endometrial carcinoma cases from January 2000-December 2010. Data was extracted from clinical charts including patients' age at the time of diagnosis, surgical stage, post-operative therapy, site of recurrence, and cause and time of death. All patients underwent surgical staging with a total hysterectomy and bilateral salpingo-oophorectomy (TAH+BSO), and pelvic washings. Pelvic and para-aortic lymphadenectomy was performed for patients with advanced stage disease and high grade tumors. Patients were treated according to the National Comprehensive Cancer Network (NCCN) guidelines (www.cancer.gov).

Histological evaluation: Tumor grade was assessed using the International Federation of Gynecology and Obstetrics (FIGO) system and by nuclear grading. FIGO grading was determined as follows; tumors with <5% solid areas were grade 1 (G1), tumors with 5%-50% solid areas were grade 2 (G2) and tumors with > 50% solid areas were grade 3 (G3). Tumors nuclear grade was determined by the variation in nuclear size and shape, chromatin distribution and size of the nucleoli. Tumor stage was assigned based on 1988 FIGO surgical staging guidelines (FIGO, 1989). [16]. All slides were examined by an expert gynecologic pathologist for confirmation of the histologic type, tumor size, tumor grade, depth of myometrial invasion (MI) and presence of lymphovascular invasion (LVI).

Immunohistochemistry: Four µm thick sections from 279 cases were deparaffinized with xylene and washed with ethanol. In addition, 5 sections from normal endometrium

were also included in the study. Sections were cooled for 20 minutes and incubated for 10 minutes with 3% H_2O_2 to quench endogenous peroxidase activity. Blocking was performed using a serum-free protein block, Dakocytomation (Carpenteria, CA), for 30 minutes. The sections were pretreated with an EDTA buffer saline solution, steamed for 20 minutes and then sections were incubated with HIF1α (monoclonal; 1:1000 dilution; Novus Biologicals, Littleton, CO-USA) and aldolaseC (monoclonal; 1:250 dilution; Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. The diaminobenzidine complex was used as a chromogen. Negative control slides omitting the primary antibody were included in all assays. The extent of immunochemical reactivity was graded based on intensity as follows: 0 (negative), 1+ (weak), 2+ (moderate), 3+ (strong). For the sake of statistical analysis, negative and weak stains were grouped as group I (negative) and moderate and strong as group II (positive).

Statistical Analyses: The clinical parameters used for modeling were age, tumor size, histologic subtype, tumor stage, myometrial depth of invasion, LVI, FIGO grade, nuclear grade, lymph node status, recurrence, recurrence time, survival time and status. To test the association between aldolaseC/HIF1α IHC (positive and negative) and the clinical parameters, Fisher's exact test was performed for categorical parameters and the logistic regression model was used for continuous ones. Pearson correlation was used to check the association of IHC between aldolaseC and HIF1α. All statistical analysis was performed using the statistical software package R (http://www.r-project.org/).

Results:

Clinical and pathological features:

279 patients diagnosed with endometrial carcinoma were included in the study. The age ranged from 29 to 97 years (median age 65 years). The follow-up period ranged from 0 (as one patient was lost for follow-up) to 137.16 months (median 46.32 months). The clinical and histological features are summarized in Table 1.

Table 1.Clinical and pathologic features of patients (data in parentheses are percentages).

Characteristics		
No. of evaluable patients		279
Age, year		
	Median	65
	Range	29-97
Follow time, months		
	Median	46.32
	Range	(0-137.16)
Stage		
	I	181(64.87)
	II	35(12.54)
	III	43(15.41)
	IV	20(7.17)
Subtype		
	Endometrioid	202(72.4)
	CCC+serous	77(27.6)
Grade(FIGO)		
	1	119(42.65)
	2	53(19)
	3	107(38.35)
Grade(Nuclear)		
	1	93(33.33)
	2	75(26.88)
	3	111(39.78)
Tumor size, cm		
	<=2	62(22)
	>2	217(78)
Depth of invasion		
	Median	28
	Range	0-100
LVI		
	N	202(72.4)

	Y	77(27.6)
Lymph node status		
	Positive	50(17.92)
	Negative	135(48.39)
	Unknown	94(33.69)
Recurrence		
	N	215(77.06)
	Y	47(16.85)
	Persistent	12(4.3)
	Progression	4(1.43)
	Unknown	1(0.36)
Status		
	Alive with no evidence of disease (ANED)	188(67.38)
	Alive with evidence of disease (AWED)	22(7.89)
	Dead of disease (DOD)	38(13.62)
	Dead with no evidence of disease (DNED)	22(7.89)
	dead unknown cause	1(0.36)
	Dead with evidence of disease(DWED)	7(2.51)
	lost for FU	1(0.36)

AldolaseC and HIF1α immunoexpressions:

The stain intensity was diffuse and homogenous throughout the tumor. The staining patterns were nuclear for HIF1α and cytoplasmic for ALDC. The 5 cases of normal endometrium all came from patients who underwent a hysterectomy for benign reasons, such as fibroids, and were weakly positive for ALDC and negative for HIF1α **(Fig 1A, 1B).** There was a strong positive association between ALDC and HIF1α proteins (p=0.0017) in endometrial cancer. The results of the association of ALDC and the clinical-pathological variables are shown in **Table 2**. 59/279 (22%) of cases were negative for ALDC protein and 220/279 (78%) were positive **(Fig 2A, 2B).** ALDC was only associated with depth of myometrial invasion (p=0.0438), lending to the conclusion that tumors that invade deeper into the myometrium are more likely to overexpress ALDC.

Table 2. Association of Aldolase IHC with Clinicopathologic Variables.

Variables	Negative (n = 59)	Positive (n = 220)	P value
Age (median)	69	65	0.4*
Myometrial invasion (median)	21	31.5	0.0438
Stage			
I	41(69.49)	140(63.64)	
II	8(13.56)	27(12.27)	0.7656
III	7(11.86)	36(16.36)	
IV	3(5.08)	17(7.73)	
Tumor Size			
<=2 cm	15(25.42)	47(21.36)	0.4866^
>2 cm	44(74.58)	173(78.64)	
LVI			
N	46(77.97)	156(70.91)	0.3273
Y	13(22.03)	64(29.09)	
Grade_FIGO			
1	24(40.68)	95(43.18)	
2	11(18.64)	42(19.09)	0.9266
3	24(40.68)	83(37.73)	
Grade_nuclear			
1	15(25.42)	78(35.45)	
2	19(32.2)	56(25.45)	0.3096
3	25(42.37)	86(39.09)	
Lymph node status			
Positive	6(16.22)	44(29.73)	0.1461
Negative	31(83.78)	104(70.27)	
Subtype			
CCC+Serous	17(28.81)	60(27.27)	0.87
Endometrioid	42(71.19)	160(72.73)	
Recurrence			
N	45(81.82)	170(82.13)	1
Y	10(18.18)	37(17.87)	
Status			
ANED	41(69.49)	147(66.82)	0.7561
Others	18(30.51)	73(33.18)	

*Pvalue calculated by logistic linear regression

^Pvalue calculated by Fisher exact test

The results of the association between HIF1α and the clinical-pathological variables are summarized in **Table 3**. 66/279 (24%) of cases did not express HIF1α and 213/279 (76%) did **(Fig 3A, 3B).** There was an association between HIF1α and histologic subtype and tumor grade (p=0.018 and 0.0368 respectively). This lead us to the conclusion that endometrioid adenocarcinomas are more likely to express HIF1α than clear cell and serous adenocarcinomas. In addition, high grade tumors, G2 and G3, are more likely to express HIF1α than low grade tumors (G1).

Table 3. Association of IHFα IHC with Clinicopathologic Variables.

Variables	Negative (n = 66)	Positive (n = 213)	P value
Age (median)	65	65	0.3571*
Myometrial invasion (median)	40	25	0.0646^
Stage			
I	39(59.09)	142(66.67)	
II	10(15.15)	25(11.74)	0.6376
III	11(16.67)	32(15.02)	
IV	6(9.09)	14(6.57)	
Tumor Size			
<=2 cm	15(22.73)	47(22.07)	1
>2 cm	51(77.27)	166(77.93)	
LVI			
N	46(69.7)	156(73.24)	0.6367
Y	20(30.3)	57(26.76)	
Grade_FIGO			
1	22(33.33)	97(45.54)	
2	9(13.64)	44(20.66)	0.0231
3	35(53.03)	72(33.8)	
Grade_nuclear			
1	19(28.79)	74(34.74)	
2	12(18.18)	63(29.58)	0.0368
3	35(53.03)	76(35.68)	
Lymph node status			
Positive	14(26.92)	36(27.07)	1

	Negative	38(73.08)	97(72.93)	
Subtype				
	CCC+Serous	26(39.39)	51(23.94)	0.018
	Endometrioid	40(60.61)	162(76.06)	
Recurrence				
	N	48(81.36)	167(82.27)	0.849
	Y	11(18.64)	36(17.73)	
Status				
	ANED	40(60.61)	148(69.48)	0.1805
	Others	26(39.39)	65(30.52)	

*Pvalue calculated by logistic linear regression
^Pvalue calculated by Fisher exact test

Finally, neither ALDC nor HIF1α proteins individually, or any combination of their expressions, (ALDC+/HIF1α-), (ALDC+/HIF1α+), (ALDC-/HIF1α+), (ALDC-/HIF1α-), had an impact on disease outcome such as recurrence, progression or death of disease.

Discussion:

HIF1α is a transcription factor and it is a major regulator of oxygen homeostasis within cells, [14]. It plays an important role in tumorigenesis through its enhancement of angiogenesis via regulation of vascular endothelial growth factor (VEGF) transcription which promotes endothelial cell migration toward a hypoxic area. In addition, in hypoxic conditions, HIF1α regulates metabolism by shifting the production of ATP via oxidative phosphorylation to anaerobic metabolism by stimulation of a variety of glycolytic enzymes, including ALDC, [17,18]. Even though this relation between HIF1α and ALDC is well established in vitro and animal models, their association in human cancer tissues, namely endometrial cancer, is still widely unexplored. Our main goal was to evaluate the expression of ALDC and HIF1α in a large series of cases of endometrial cancer. We found that ALDC and HIF1α were both expressed in the majority of endometrial cancer cases and they were negative in normal endometrium. In addition, a strong positive association between HIF1α and ALDC was seen in these cases.

Previously, using cDNA microarray, we showed that one of the genes that is upregulated in uterine serous carcinoma (USC) in comparison to endometrioid adenocarcinoma (EAC) is *aldolaseC,* [19, 20]. Furthermore, qRT-PCR showed that ALDC mRNA level was overexpressed in endometrial carcinomas in comparison to normal endometrium, but there was no association between ALDC-mRNA level and the endometrial cancer subtypes, [21]. Similarly, in this

study, we found that ALDC was not associated with tumor subtype. However, it was associated with one of the most reliable pathological prognostic factors of poor outcome, depth of myometrial invasion. In addition, we found that overexpression of HIF1α is associated with high tumor grade, another major prognostic factor of poor outcome in patients with EC. This further confirms that ALDC and HIF1α overexpression may be related to tumor aggressiveness, [15,22]. All the above data leads us to suggest that these two proteins may be key regulators in endometrial carcinogenesis.

Recently, with clearer understanding of the function of HIF1α and its pathway, efforts directed at manipulation of this complex in order to decrease cellular HIF1α levels in tumor cells have been undertaken. Thus, modulation of HIF1α and its pathway promises to have a significant impact on cancer and it seems to be an attractive therapeutic option for patients with endometrial cancer.

In summary, ALDC and HIF1α seem to play a role in the tumorigenesis of endometrial cancer and their expression may be an indication of tumor aggressiveness.

Legends:

Fig 1A: ALDC is negative in normal endometrium (x 40) and **Fig 1B.** HIF-1α is negative/weakly positive in normal endometrium (x40).

Fig 2A: ALDC in endometrioid adenocarcinoma and in serous adenocarcinoma **(Fig 2B).** The staining is strong and cytoplasmic (x40).

Fig 3A: HIF-1α in endometrioid adenocarcinoma and in serous adenocarcinoma **(Fig 3B).** The staining is strong and it has a nuclear pattern (x40).

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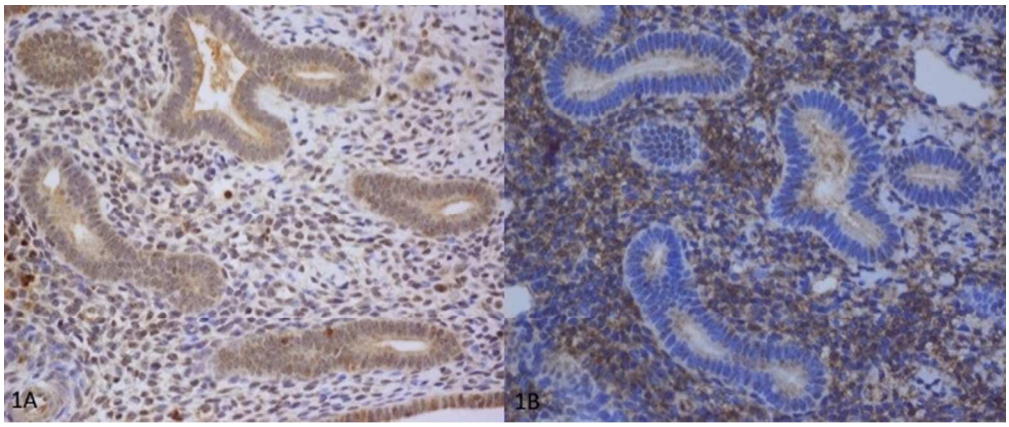

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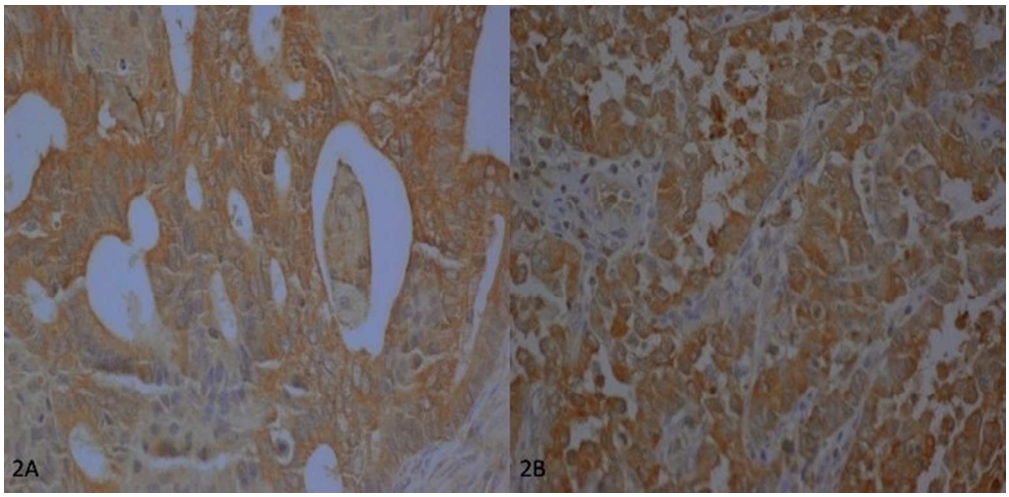

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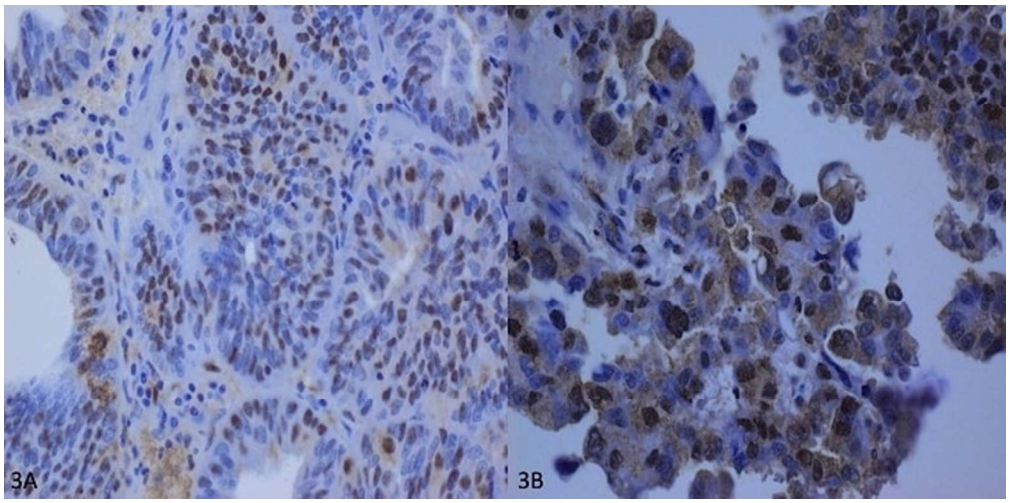

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The role of Hypoxic-inducible Factor (HIF1α) and Aldolase C Protein in Endometrial Carcinogenesis: A retrospective Study of 279 Patients.

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The role of Hypoxic-inducible Factor (HIF1α) and Aldolase C Protein in Endometrial Carcinogenesis: A retrospective Study of 279 Patients.

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Abstract:

Objectives: Hypoxia-inducible factor (HIF1α) plays an integral role in response to hypoxia, controlling dozens of target genes including aldolaseC (ALDC), an important enzyme in the glycolytic pathway. It also induces angiogenesis, allowing survival and proliferation of cancer cells. Thus, inhibition of HIF1α may be an attractive therapeutic option. The aims of our study were to (1) evaluate the expressions of HIF1α and ALDC in patients with endometrial cancer (EC) and define their association with disease outcome and (2) determine the existence of an association between HIF1α and ALDC proteins. **Design:** We performed immunohistochemistry using antibodies to ALDC and HIF1α on paraffin-embedded tissue from 279 patients. The association of ALDC and HIF1α immunoexpression was evaluated, as well as the association of their expressions with the following clinical parameters; age, histologic subtype, myometrial depth of invasion, lymphovascular invasion, FIGO grade, lymph nodes status, and disease status. **Results:** ALDC and HIF1α were overexpressed in the vast majority of EC cases (78% and 76% respectively). There was a strong positive association between HIF1α and ALDC (p=0.0017). There was a significant association between ALDC and depth of myometrial invasion (p=0.0438), and between HIF1α and tumor grade (p= 0.0231) and tumor subtype (p=0.018). However, there was no association between either ALDC nor HIF1α and disease status. **Conclusions:** ALDC and HIF1α play an important role in endometrial carcinogenesis. Their expression by the majority of EC makes inhibition of HIF1α a very attractive therapeutic option for treating patients with EC and we suggest that will be prospectively validated in future studies.

Key words: HIF1α; AldolaseC; Immunohistochemistry; Endometrial cancer; Disease outcome.

Subject headings: HIF1α and aldolaseC in endometrial cancer

Article Summary:

1- Article focus:

- HIF1 α and aldolase C expressions in patients with endometrial cancer
- HIF1 α and aldolase C interaction in vivo in endometrial cancer.
- HI1 α and aldolaseC value in predicting disease outcome in patients with endometrial cancer.

2- Key messages:

- HIF1 α and aldolase C are frequently expressed in endometrial cancer.
- There is strong association between HIF1 α and aldolase C in endometrial cancer and therefore they could play an important role in its pathogenesis.
- HIF1 α and aldolase C are associated with poor prognostic factors.
- HIF1 α and aldolase C are not independent predictive biomarkers of poor outcome.

3- Strengths:

- It is a large study of 279 patients.
- It is one of the few in the literature.

- It is the first to evaluate the association of HIF1 α and aldolase C in vivo in endometrial cancer.

Limitations:

- The study did not have a large numbers of type II cancers (serous and clear cell carcinomas).
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Funding statement:

We, the authors, declare that there was no funding for this study.

Competing interests:

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Contribution statement:

PMF: designed and wrote the study

DW, SL: performed the statistical analysis

DS, HG, TM: reviewed the patients charts

DS, FO: helped in reviewing the manuscript.

SL (Shashikant Lele), TP: Conducting and writing the study

Introduction:

Endometrial cancer (EC) is the most common gynecologic malignancy in developed countries. There are approximately 42,000 cases diagnosed annually in the United States, resulting in almost 8,000 deaths, [1]. EC has been classified into two types based on morphology, pathogenesis, behavior and treatment: type I (endometrioid and mucinous carcinomas), and type II (serous and clear cell carcinomas). Type I is usually low-grade and low stage at initial presentation. Type II is usually high grade and advanced stage at initial presentation. The most reliable prognostic factors in predicting disease outcome in EC are tumor grade, tumor stage, tumor subtype, depth of myometrial invasion, and lymph node involvement, [2-4].

One of the most prominent metabolic alterations in cancer cells is an increase in aerobic glycolysis, known as the Warburg effect after its discovery by Otto Warburg in 1920, [5]. This increase in glycolysis, due to a shift in glucose metabolism from oxidative phosphorylation into the aerobic glycolysis pathway, provides the tumor with metabolic and survival advantages, [6-8]. Aldolase, a critical enzyme in the glycolytic pathway, catalyses the reversible conversion of fructose-1,6-biphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Aldolase has three distinct isoenzymes, A, B, and C, which are similar in sequence with 78% identity between A and C and 68% identity between B and C, [9,10]. Originally identified in brain tissue, aldolaseC (ALDC) has been seen to be overexpressed in carcinomas of the lung, kidney, cervix and endometrium, [11-13].

The Hypoxic-inducible factor (HIF1) gene codes for two subunits, α and β, and is usually activated by hypoxic conditions, a microenvironment that commonly accompanies cancerous tumors. When activated, HIF1 can interact with enzymes and other transcription factors in order to control vascularization and tissue growth. HIF1α was recently identified as a potent regulator of ALDC, another mechanism by which it may promote carcinogenesis, [14]. Thus, attempts to target the HIF1α pathway in hopes of suppressing cancer cell proliferation and progression are underway. In the gynecologic tract, HIF1α expression increases as the endometrium undergoes changes from normal to premalignant to endometrioid adenocarcinoma. This is paralleled by increased angiogenesis in the endometrium suggesting that HIF1α might be a key regulator in endometrial carcinogenesis, [15].

Though the interaction between HIF1α and ALDC has been seen in vivo, their interaction in human samples and in endometrial carcinoma has not yet been described. Therefore, the aims of this study are (1) to evaluate the expression of HIF1α and ALDC proteins in patients with endometrial cancer and to find an association between these two proteins in this patient sample and (2)- to determine whether either of these two proteins independently or any combination of their expressions might have an impact on disease outcome.

Materials and Methods:

Patient population: After obtaining IRB approval, the pathology archives were searched for endometrial carcinoma cases from January 2000-December 2010. Data was extracted from clinical charts including patients' age at the time of diagnosis, surgical stage, post-operative therapy, site of recurrence, and cause and time of death. All patients underwent surgical staging with a total hysterectomy and bilateral salpingo-oophorectomy (TAH+BSO), and pelvic washings. Pelvic and para-aortic lymphadenectomy was performed for patients with advanced stage disease and high grade tumors. Patients were treated according to the National Comprehensive Cancer Network (NCCN) guidelines (www.cancer.gov).

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Immunohistochemistry: Four µm thick sections from 279 cases were deparaffinized with xylene and washed with ethanol. In addition, 5 sections from normal endometrium were also included in the study. Sections were cooled for 20 minutes and incubated for 10 minutes with 3% H_2O_2 to quench endogenous peroxidase activity. Blocking was performed using a serum-free protein block, Dakocytomation (Carpenteria, CA), for 30 minutes. The sections were pretreated with an EDTA buffer saline solution, steamed for 20 minutes and then sections were incubated with HIF1α (monoclonal; 1:1000 dilution; Novus Biologicals, Littleton, CO-USA) and aldolaseC (monoclonal; 1:250 dilution; Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. The diaminobenzidine complex was used as a chromogen. Negative control slides omitting the primary antibody were included in all assays. Breast cancer was used as positive controls for HIF1 α and ALDC. The extent of immunochemical reactivity was graded based on intensity as follows: 0 (negative), 1+ (weak), 2+ (moderate), 3+ (strong). For the sake of statistical analysis, negative and weak stains were grouped as group I (negative) and moderate and strong as group II (positive).

Statistical Analyses: The clinical parameters used for modeling were age, tumor size, histologic subtype, tumor stage, myometrial depth of invasion, LVI, FIGO grade, nuclear grade, lymph node status, recurrence, recurrence time, survival time and status. To test the association between aldolaseC/HIF1α IHC (positive and negative) and the clinical parameters, Fisher's exact test was performed for categorical parameters and the logistic regression model was used for continuous ones. Pearson correlation was used to check

the association of IHC between aldolaseC and HIF1α. All statistical analysis was performed using the statistical software package R (http://www.r-project.org/).

Results:

Clinical and pathological features:

279 patients diagnosed with endometrial carcinoma were included in the study. The age ranged from 29 to 97 years (median age 65 years). The follow-up period ranged from 0 (as one patient was lost for follow-up) to 137.16 months (median 46.32 months). The clinical and histological features are summarized in Table 1.

Table 1.Clinical and pathologic features of patients (data in parentheses are percentages).

Characteristics		
No. of evaluable patients		279
Age, year		
	Median	65
	Range	29-97
Follow time, months		
	Median	46.32
	Range	(0-137.16)
Stage		
	I	181(64.87)
	II	35(12.54)
	III	43(15.41)
	IV	20(7.17)
Subtype		
	Endometrioid	202(72.4)
	CCC+serous	77(27.6)
Grade(FIGO)		
	1	119(42.65)
	2	53(19)
	3	107(38.35)
Grade(Nuclear)		
	1	93(33.33)
	2	75(26.88)
	3	111(39.78)
Tumor size, cm		
	<=2	62(22)
	>2	217(78)
Depth of invasion		
	Median	28
	Range	0-100
LVI		
	N	202(72.4)

	Y	77(27.6)
Lymph node status		
	Positive	50(17.92)
	Negative	135(48.39)
	Unknown	94(33.69)
Recurrence		
	N	215(77.06)
	Y	47(16.85)
	Persistent	12(4.3)
	Progression	4(1.43)
	Unknown	1(0.36)
Status		
	Alive with no evidence of disease (ANED)	188(67.38)
	Alive with evidence of disease (AWED)	22(7.89)
	Dead of disease (DOD)	38(13.62)
	Dead with no evidence of disease (DNED)	22(7.89)
	dead unknown cause	1(0.36)
	Dead with evidence of disease(DWED)	7(2.51)
	lost for FU	1(0.36)

AldolaseC and HIF1α immunoexpressions:

The staining patterns were nuclear for HIF1α and cytoplasmic for ALDC. The 5 cases of normal endometrium all came from patients who underwent a hysterectomy for benign reasons, such as fibroids, and were weakly positive for ALDC and negative for HIF1α **(Fig 1A, 1B).** There was a strong positive association between ALDC and HIF1α proteins (p=0.0017) in endometrial cancer. The results of the association of ALDC and the clinical-pathological variables are shown in **Table 2**. 59/279 (22%) of cases were negative for ALDC protein and 220/279 (78%) were positive **(Fig 2A, 2B).** ALDC was only associated with depth of myometrial invasion (p=0.0438), lending to the conclusion that tumors that invade deeper into the myometrium are more likely to overexpress ALDC.

Table 2. Association of AldolaseC IHC with Clinicopathologic Variables.

Variables	Negative	Positive	P value

		(n = 59)	(n = 220)	
Age (median)		69	65	0.4*
Myometrial invasion (median)		21	31.5	0.0438
Stage				
	I	41(69.49)	140(63.64)	0.7656
	II	8(13.56)	27(12.27)	
	III	7(11.86)	36(16.36)	
	IV	3(5.08)	17(7.73)	
Tumor Size				
	<=2 cm	15(25.42)	47(21.36)	0.4866^
	>2 cm	44(74.58)	173(78.64)	
LVI				
	N	46(77.97)	156(70.91)	0.3273
	Y	13(22.03)	64(29.09)	
Grade_FIGO				
	1	24(40.68)	95(43.18)	0.9266
	2	11(18.64)	42(19.09)	
	3	24(40.68)	83(37.73)	
Grade_nuclear				
	1	15(25.42)	78(35.45)	0.3096
	2	19(32.2)	56(25.45)	
	3	25(42.37)	86(39.09)	
Lymph node status				
	Positive	6(16.22)	44(29.73)	0.1461
	Negative	31(83.78)	104(70.27)	
Subtype				
	CCC+Serous	17(28.81)	60(27.27)	0.87
	Endometrioid	42(71.19)	160(72.73)	
Recurrence				
	N	45(81.82)	170(82.13)	1
	Y	10(18.18)	37(17.87)	
Status				
	ANED	41(69.49)	147(66.82)	0.7561
	Others	18(30.51)	73(33.18)	

*Pvalue calculated by logistic linear regression

^Pvalue calculated by Fisher exact test

The results of the association between HIF1α and the clinical-pathological variables are summarized in **Table 3**. 66/279 (24%) of cases did not express HIF1α and 213/279 (76%) did **(Fig 3A, 3B).** There was an association between HIF1α and histologic subtype and tumor grade (p=0.018 and 0.0368 respectively). This lead us to the conclusion that endometrioid adenocarcinomas are more likely to express HIF1α than clear cell and serous adenocarcinomas. In addition, high grade tumors, G2 and G3, are more likely to express HIF1α than low grade tumors (G1).

Table 3. Association of IHF1α IHC with Clinicopathologic Variables.

Variables	Negative (n = 66)	Positive (n = 213)	P value
Age (median)	65	65	0.3571*
Myometrial invasion (median)	40	25	0.0646^
Stage			
I	39(59.09)	142(66.67)	
II	10(15.15)	25(11.74)	0.6376
III	11(16.67)	32(15.02)	
IV	6(9.09)	14(6.57)	
Tumor Size			
<=2 cm	15(22.73)	47(22.07)	1
>2 cm	51(77.27)	166(77.93)	
LVI			
N	46(69.7)	156(73.24)	0.6367
Y	20(30.3)	57(26.76)	
Grade_FIGO			
1	22(33.33)	97(45.54)	
2	9(13.64)	44(20.66)	0.0231
3	35(53.03)	72(33.8)	
Grade_nuclear			
1	19(28.79)	74(34.74)	
2	12(18.18)	63(29.58)	0.0368
3	35(53.03)	76(35.68)	
Lymph node status			
Positive	14(26.92)	36(27.07)	1

	Negative	38(73.08)	97(72.93)	
Subtype				
	CCC+Serous	26(39.39)	51(23.94)	0.018
	Endometrioid	40(60.61)	162(76.06)	
Recurrence				
	N	48(81.36)	167(82.27)	0.849
	Y	11(18.64)	36(17.73)	
Status				
	ANED	40(60.61)	148(69.48)	0.1805
	Others	26(39.39)	65(30.52)	

*Pvalue calculated by logistic linear regression
^Pvalue calculated by Fisher exact test

Finally, neither ALDC nor HIF1α proteins individually, or any combination of their expressions, (ALDC+/HIF1α-), (ALDC+/HIF1α+), (ALDC-/HIF1α+), (ALDC-/HIF1α-), had an impact on disease outcome such as recurrence, progression or death of disease.

Discussion:

HIF1α is a transcription factor and it is a major regulator of oxygen homeostasis within cells, [14]. It plays an important role in tumorigenesis through its enhancement of angiogenesis via regulation of vascular endothelial growth factor (VEGF) transcription which promotes endothelial cell migration toward a hypoxic area. In addition, in hypoxic conditions, HIF1α regulates metabolism by shifting the production of ATP via oxidative phosphorylation to aerobic metabolism by stimulation of a variety of glycolytic enzymes, including ALDC, [17,18]. Even though this relation between HIF1α and ALDC is well established in vitro and animal models, their association in human cancer tissues, namely endometrial cancer, is still widely unexplored. Our main goal was to evaluate the expression of ALDC and HIF1α in a large series of cases of endometrial cancer. We found that ALDC and HIF1α were both expressed in the majority of endometrial cancer cases and they were negative in normal endometrium. In addition, a strong positive association between HIF1α and ALDC was seen in these cases.

Previously, using cDNA microarray, we showed that one of the genes that is upregulated in uterine serous carcinoma (USC) in comparison to endometrioid adenocarcinoma (EAC) is *aldolaseC,* [19, 20]. Furthermore, qRT-PCR showed that ALDC mRNA level was overexpressed in endometrial carcinomas in comparison to normal endometrium, but there was no association between ALDC-mRNA level and the endometrial cancer subtypes[21]. Similarly, in this

study, we found that ALDC was not associated with tumor subtype. However, it was associated with one of the most reliable pathological prognostic factors of poor outcome, depth of myometrial invasion. In addition, we found that overexpression of HIF1α is associated with high tumor grade, another major prognostic factor of poor outcome in patients with EC. This further confirms that ALDC and HIF1α overexpression may be related to tumor aggressiveness, [15,22]. All the above data leads us to suggest that these two proteins may be key regulators in endometrial carcinogenesis.

Recently, with clearer understanding of the function of HIF1α and its pathway, efforts directed at manipulation of this complex in order to decrease cellular HIF1α levels in tumor cells have been undertaken. Thus, modulation of HIF1α and its pathway promises to have a significant impact on cancer and it seems to be an attractive therapeutic option for patients with endometrial cancer. In summary, ALDC and HIF1α seem to play a role in the tumorigenesis of endometrial cancer and their expression may be an indication of tumor aggressiveness, and we suggest that prospectively will be validated in future studies.

Legends:

Fig 1A: ALDC is negative in normal endometrium (x 40) and **Fig 1B.** HIF-1α is negative/weakly positive in normal endometrium (x40).

Fig 2A: ALDC in endometrioid adenocarcinoma and in serous adenocarcinoma **(Fig 2B).** The staining is strong and cytoplasmic (x40).

Fig 3A: HIF-1α in endometrioid adenocarcinoma and in serous adenocarcinoma **(Fig 3B).** The staining is strong and it has a nuclear pattern (x40).

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The role of Hypoxic-inducible Factor (HIF1α) and Aldolase C Protein in ~~Immunoexpression in~~ Endometrial Carcinogenesis ~~ancer~~

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Abstract:

Objectives: Hypoxia-inducible factor (HIF1α) plays an integral role in response to hypoxia, controlling dozens of target genes including aldolaseC (ALDC), an important enzyme in the glycolytic pathway. It also induces angiogenesis, allowing survival and proliferation of cancer cells. Thus, inhibition of HIF1α may be an attractive therapeutic option. The aims of our study were to (1) evaluate the expressions of HIF1α and ALDC in patients with endometrial cancer (EC) and define their association with disease outcome and (2) determine the existence of an association between HIF1α and ALDC proteins. **Design:** We performed immunohistochemistry using antibodies to ALDC and HIF1α on paraffin-embedded tissue from 279 patients. The association of ALDC and HIF1α immunoexpression was evaluated, as well as the association of their expressions with the following clinical parameters; age, histologic subtype, myometrial depth of invasion, lymphovascular invasion, FIGO grade, lymph nodes status, and disease status. **Results:** ALDC and HIF1α were overexpressed in the vast majority of EC cases (78% and 76% respectively). There was a strong positive association between HIF1α and ALDC (p=0.0017). There was a significant association between ALDC and depth of myometrial invasion (p=0.0438), and between HIF1α and tumor grade (p= 0.0231) and tumor subtype (p=0.018). However, there was no association between either ALDC nor HIF1α and disease status. **Conclusions:** ALDC and HIF1α play an important role in endometrial carcinogenesis. Their expression by the majority of EC makes inhibition of HIF1α a very attractive therapeutic option for treating patients with EC and should be confirmed by future studies.

2

Key words: HIF1α; AldolaseC; Immunohistochemistry; Endometrial cancer; Disease outcome.

Subject headings: HIF1α and aldolaseC in endometrial cancer

Article Summary:

1- Article focus:

- HIF1 α and aldolase C expressions in patients with endometrial cancer
- HIF1 α and aldolase C interaction in vivo in endometrial cancer.
- HI1 α and aldolaseC value in predicting disease outcome in patients with endometrial cancer.

2- Key messages:

- HIF1 α and aldolase C are frequently expressed in endometrial cancer.
- There is strong association between HIF1 α and aldolase C in endometrial cancer and therefore they could play an important role in its pathogenesis.
- HIF1 α and aldolase C are associated with poor prognostic factors.
- HIF1 α and aldolase C are not independent predictive biomarkers of poor outcome.

3- Strengths:

- It is a large study of 279 patients.
- It is one of the few in the literature.

- It is the first to evaluate the association of HIF1 α and aldolase C in vivo in endometrial cancer.

Limitations:

- The study did not have a large numbers of type II cancers (serous and clear cell carcinomas).
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PMF: designed and wrote the study

DW, SL: performed the statistical analysis

DS, HG, TM: reviewed the patients charts

DS, FO: helped in reviewing the manuscript.

SL (Shashikant Lele), TP: ~~mentored and were consulted in designing,~~ Cconducting and writing the study

Introduction:

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Age, year		
	Median	65
	Range	29-97
Follow time, months		
	Median	46.32
	Range	(0-137.16)
Stage		
	I	181(64.87)
	II	35(12.54)
	III	43(15.41)
	IV	20(7.17)
Subtype		
	Endometrioid	202(72.4)
	CCC+serous	77(27.6)
Grade(FIGO)		
	1	119(42.65)
	2	53(19)
	3	107(38.35)
Grade(Nuclear)		
	1	93(33.33)
	2	75(26.88)
	3	111(39.78)
Tumor size, cm		
	<=2	62(22)
	>2	217(78)
Depth of invasion		
	Median	28
	Range	0-100
LVI		
	N	202(72.4)

	Y	77(27.6)
Lymph node status		
	Positive	50(17.92)
	Negative	135(48.39)
	Unknown	94(33.69)
Recurrence		
	N	215(77.06)
	Y	47(16.85)
	Persistent	12(4.3)
	Progression	4(1.43)
	Unknown	1(0.36)
Status		
	Alive with no evidence of disease (ANED)	188(67.38)
	Alive with evidence of disease (AWED)	22(7.89)
	Dead of disease (DOD)	38(13.62)
	Dead with no evidence of disease (DNED)	22(7.89)
	dead unknown cause	1(0.36)
	Dead with evidence of disease(DWED)	7(2.51)
	lost for FU	1(0.36)

AldolaseC and HIF1α immunoexpressions:

~~The stain intensity was diffuse and homogenous throughout the tumor.~~ The staining patterns were nuclear for HIF1α and cytoplasmic for ALDC. The 5 cases of normal endometrium all came from patients who underwent a hysterectomy for benign reasons, such as fibroids, and were weakly positive for ALDC and negative for HIF1α **(Fig 1A, 1B).** There was a strong positive association between ALDC and HIF1α proteins (p=0.0017) in endometrial cancer. The results of the association of ALDC and the clinical-pathological variables are shown in **Table 2**. 59/279 (22%) of cases were negative for ALDC protein and 220/279 (78%) were positive **(Fig 2A, 2B).** ALDC was only associated with depth of myometrial invasion (p=0.0438), lending to the conclusion that tumors that invade deeper into the myometrium are more likely to overexpress ALDC.

Table 2. Association of AldolaseC IHC with Clinicopathologic Variables.

Variables	Negative (n = 59)	Positive (n = 220)	P value
Age (median)	69	65	0.4*
Myometrial invasion (median)	21	31.5	0.0438
Stage			
I	41(69.49)	140(63.64)	
II	8(13.56)	27(12.27)	0.7656
III	7(11.86)	36(16.36)	
IV	3(5.08)	17(7.73)	
Tumor Size			
<=2 cm	15(25.42)	47(21.36)	0.4866^
>2 cm	44(74.58)	173(78.64)	
LVI			
N	46(77.97)	156(70.91)	0.3273
Y	13(22.03)	64(29.09)	
Grade_FIGO			
1	24(40.68)	95(43.18)	
2	11(18.64)	42(19.09)	0.9266
3	24(40.68)	83(37.73)	
Grade_nuclear			
1	15(25.42)	78(35.45)	
2	19(32.2)	56(25.45)	0.3096
3	25(42.37)	86(39.09)	
Lymph node status			
Positive	6(16.22)	44(29.73)	0.1461
Negative	31(83.78)	104(70.27)	
Subtype			
CCC+Serous	17(28.81)	60(27.27)	0.87
Endometrioid	42(71.19)	160(72.73)	
Recurrence			
N	45(81.82)	170(82.13)	1
Y	10(18.18)	37(17.87)	
Status			
ANED	41(69.49)	147(66.82)	0.7561
Others	18(30.51)	73(33.18)	

*Pvalue calculated by logistic linear regression

^Pvalue calculated by Fisher exact test

The results of the association between HIF1α and the clinical-pathological variables are summarized in **Table 3**. 66/279 (24%) of cases did not express HIF1α and 213/279 (76%) did **(Fig 3A, 3B).** There was an association between HIF1α and histologic subtype and tumor grade (p=0.018 and 0.0368 respectively). This lead us to the conclusion that endometrioid adenocarcinomas are more likely to express HIF1α than clear cell and serous adenocarcinomas. In addition, high grade tumors, G2 and G3, are more likely to express HIF1α than low grade tumors (G1).

Table 3. Association of IHF1α IHC with Clinicopathologic Variables.

Variables	Negative (n = 66)	Positive (n = 213)	P value
Age (median)	65	65	0.3571*
Myometrial invasion (median)	40	25	0.0646^
Stage			
I	39(59.09)	142(66.67)	
II	10(15.15)	25(11.74)	0.6376
III	11(16.67)	32(15.02)	
IV	6(9.09)	14(6.57)	
Tumor Size			
<=2 cm	15(22.73)	47(22.07)	1
>2 cm	51(77.27)	166(77.93)	
LVI			
N	46(69.7)	156(73.24)	0.6367
Y	20(30.3)	57(26.76)	
Grade_FIGO			
1	22(33.33)	97(45.54)	
2	9(13.64)	44(20.66)	0.0231
3	35(53.03)	72(33.8)	
Grade_nuclear			
1	19(28.79)	74(34.74)	
2	12(18.18)	63(29.58)	0.0368
3	35(53.03)	76(35.68)	
Lymph node status			
Positive	14(26.92)	36(27.07)	1

	Negative	38(73.08)	97(72.93)	
Subtype				
	CCC+Serous	26(39.39)	51(23.94)	0.018
	Endometrioid	40(60.61)	162(76.06)	
Recurrence				
	N	48(81.36)	167(82.27)	0.849
	Y	11(18.64)	36(17.73)	
Status				
	ANED	40(60.61)	148(69.48)	0.1805
	Others	26(39.39)	65(30.52)	

*Pvalue calculated by logistic linear regression
^Pvalue calculated by Fisher exact test

Finally, neither ALDC nor HIF1α proteins individually, or any combination of their expressions, (ALDC+/HIF1α-), (ALDC+/HIF1α+), (ALDC-/HIF1α+), (ALDC-/HIF1α-), had an impact on disease outcome such as recurrence, progression or death of disease.

Discussion:

HIF1α is a transcription factor and it is a major regulator of oxygen homeostasis within cells, [14]. It plays an important role in tumorigenesis through its enhancement of angiogenesis via regulation of vascular endothelial growth factor (VEGF) transcription which promotes endothelial cell migration toward a hypoxic area. In addition, in hypoxic conditions, HIF1α regulates metabolism by shifting the production of ATP via oxidative phosphorylation to a~~na~~erobic metabolism by stimulation of a variety of glycolytic enzymes, including ALDC, [17,18]. Even though this relation between HIF1α and ALDC is well established in vitro and animal models, their association in human cancer tissues, namely endometrial cancer, is still widely unexplored. Our main goal was to evaluate the expression of ALDC and HIF1α in a large series of cases of endometrial cancer. We found that ALDC and HIF1α were both expressed in the majority of endometrial cancer cases and they were negative in normal endometrium. In addition, a strong positive association between HIF1α and ALDC was seen in these cases.

Previously, using cDNA microarray, we showed that one of the genes that is upregulated in uterine serous carcinoma (USC) in comparison to endometrioid adenocarcinoma (EAC) is *aldolaseC,* [19, 20]. Furthermore, qRT-PCR showed that ALDC mRNA level was overexpressed in endometrial carcinomas in comparison to normal endometrium, but there was no association between ALDC-mRNA level and the endometrial cancer subtypes~~,~~ [21]. Similarly, in this

study, we found that ALDC was not associated with tumor subtype. However, it was associated with one of the most reliable pathological prognostic factors of poor outcome, depth of myometrial invasion. In addition, we found that overexpression of HIF1α is associated with high tumor grade, another major prognostic factor of poor outcome in patients with EC. This further confirms that ALDC and HIF1α overexpression may be related to tumor aggressiveness, [15,22]. All the above data leads us to suggest that these two proteins may be key regulators in endometrial carcinogenesis.

Recently, with clearer understanding of the function of HIF1α and its pathway, efforts directed at manipulation of this complex in order to decrease cellular HIF1α levels in tumor cells have been undertaken. Thus, modulation of HIF1α and its pathway promises to have a significant impact on cancer and it seems to be an attractive therapeutic option for patients with endometrial cancer.

In summary, ALDC and HIF1α seem to play a role in the tumorigenesis of endometrial cancer and their expression may be an indication of tumor aggressiveness, -and we suggest that prospectively will be validated in future studies.

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Legends:

Fig 1A: ALDC is negative in normal endometrium (x 40) and **Fig 1B.** HIF-1α is negative/weakly positive in normal endometrium (x40).

Fig 2A: ALDC in endometrioid adenocarcinoma and in serous adenocarcinoma **(Fig 2B).** The staining is strong and cytoplasmic (x40).

Fig 3A: HIF-1α in endometrioid adenocarcinoma and in serous adenocarcinoma **(Fig 3B).** The staining is strong and it has a nuclear pattern (x40).

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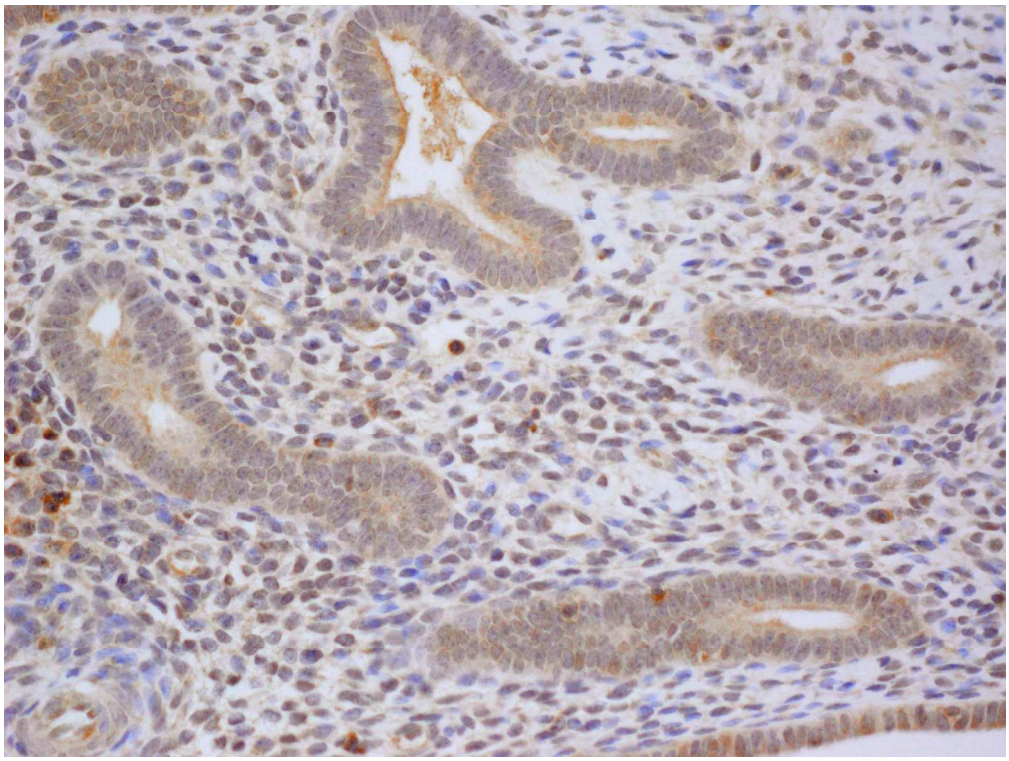

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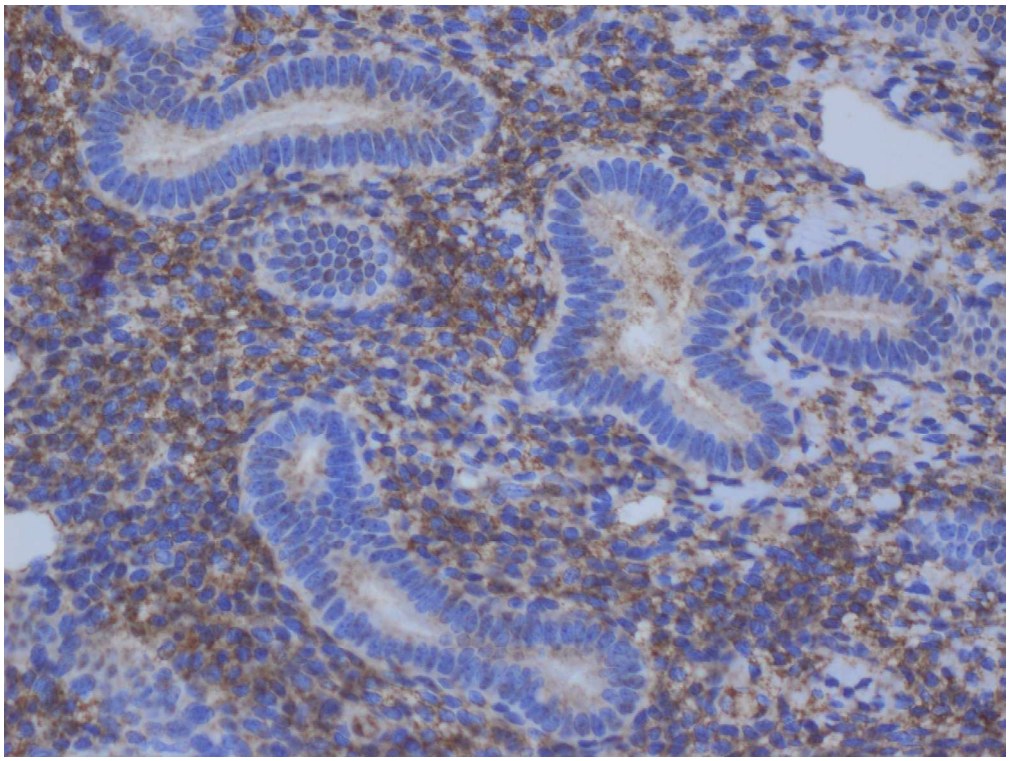

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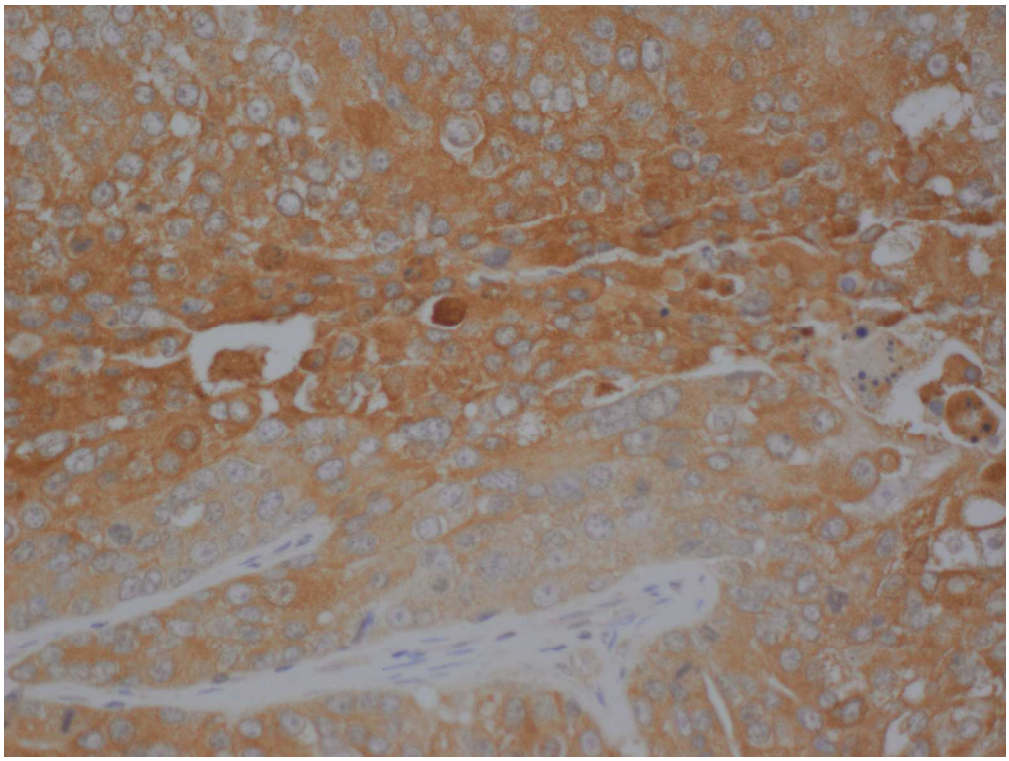

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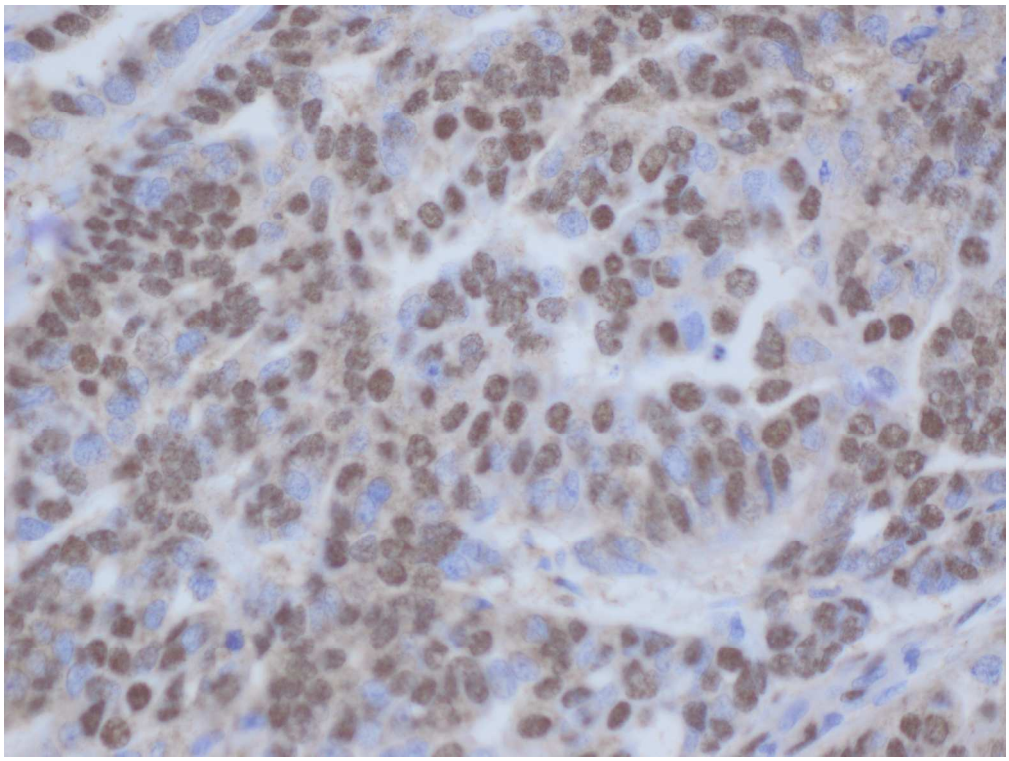

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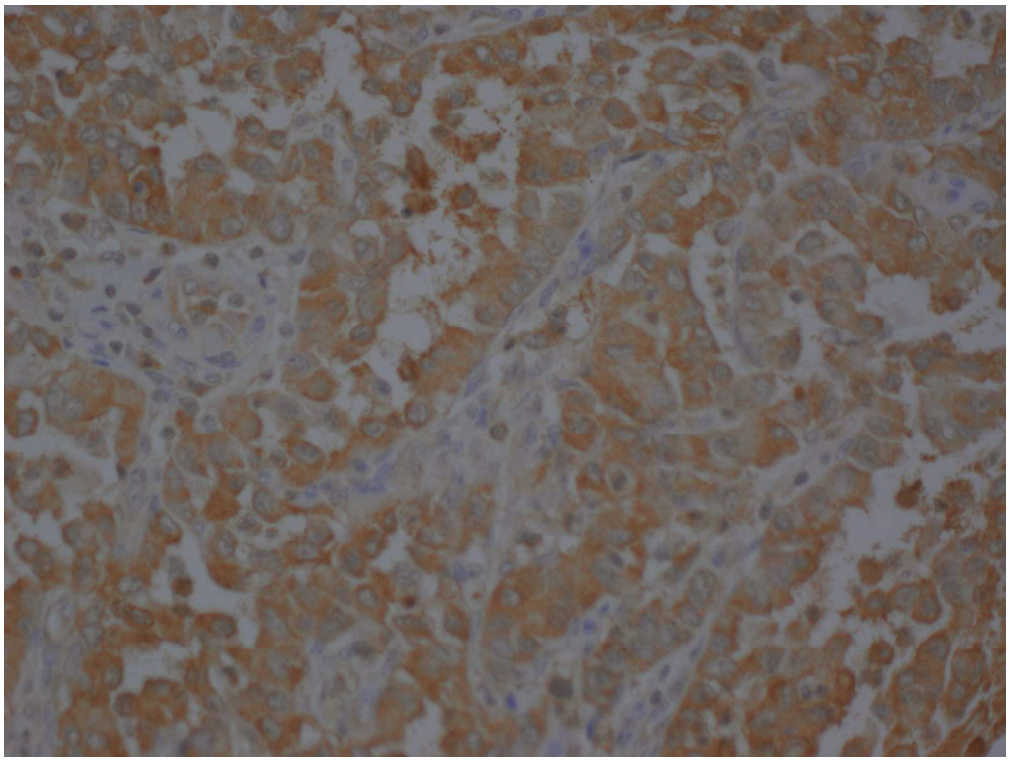

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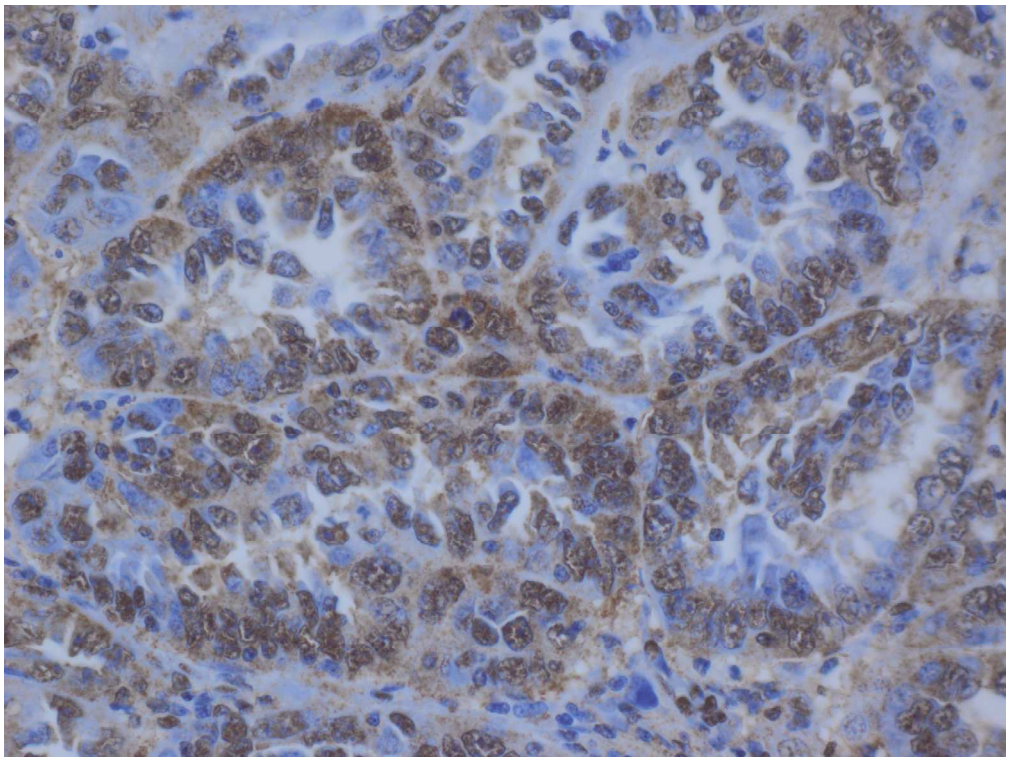

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