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Expression of Membrane Type 1 Matrix Metalloproteinase (MMP-14) in Epithelial Ovarian Cancer: High Level Expression in Clear Cell Carcinoma

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Abstract

Objective—Clear cell carcinomas of the ovary constitute approximately 5% of all ovarian neoplasms and have a distinct gene expression profile relative to other ovarian carcinoma histotypes. Tumors often present as an early stage large pelvic mass with a high degree of recurrence and frequent early metastasis. Matrix metalloproteinases (MMPs) play a role in intraperitoneal metastasis through breakdown of cell-cell and cell-matrix barriers, enabling anchoring of secondary lesions and promoting proliferation in a geometrically constrained matrix environment. The objective of this study was to evaluate MMP expression in ovarian clear cell carcinoma.

Methods—Immunohistochemistry was used to evaluate expression of membrane type 1 MMP (MMP-14), MMP-2 and MMP-9 in a panel of ovarian tumors. Western blotting and gelatin zymography were used to examine MMP-14 expression and activity in the clear cell carcinoma cell line ES2. The ability of ES2 cells to invade and proliferate within three-dimensional collagen gels was evaluated.

Results—High level expression of MMP-14 and MMP-2 were observed in ovarian clear cell carcinoma relative to other histotypes (94-95% strong positive). MMP-14 was expressed and active in cultured ES2 cells. ES2 cells also exhibited MMP-dependent invasion of and proliferation within three-dimensional collagen gels.

Conclusions—The high level expression of MMP-14 together with *in vitro* functional analyses suggest that MMP-14 may contribute to both the proliferative capacity and the enhanced parenchymal metastasis of ovarian clear cell carcinoma.

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Keywords

ovarian clear cell carcinoma; matrix metalloprotease (MMP); MMP-14; MMP-2; invasion; metastasis

Introduction

Ovarian cancer is the major cause of death from gynecologic malignancy in the United States, resulting in over 15,000 deaths/year [1], as most patients succumb to metastatic disease. Tumors exhibit marked heterogeneity and have been classified into serous, mucinous, endometrioid, and clear cell on the basis of morphology [2-4]. More recently, large scale gene expression analyses have demonstrated that clear cell carcinomas exhibit a distinct gene expression profile, with upregulation of genes involved in drug detoxification, chemoresistance, and growth signaling [2,5,6]. Clear cell carcinomas of the ovary constitute approximately 5% of all ovarian neoplasms. Clinically, clear cell carcinomas often present as a large pelvic mass, the majority of which are detected at early stage (FIGO stage I) [7-9]. Despite the early stage diagnosis, survival rates are significantly lower for women with clear cell carcinoma relative to stage-matched serous adenocarcinoma of the ovary [9,10]. Furthermore, tumors are more chemoresistant [11], resulting in a high degree of recurrence [7] and exhibit more frequent early metastasis to lymph nodes and parenchymal organs [12].

Matrix metalloproteinases (MMPs) have been implicated in a large variety of biologic processes resulting from proteolysis of structural and signaling components of the extracellular matrix and cell surface, thereby influencing differentiation, migration, tissue invasion, and proliferation [reviewed in 13,14]. The membrane-anchored collagenase designated membrane type 1 MMP (MT1-MMP, MMP-14) is overexpressed in malignant ovarian tumors [15-20] and expression correlates with poor survival [16-18]. MMP-14 forms a trimolecular complex on the cell surface containing tissue inhibitor of metalloproteinases 2 (TIMP-2) and proMMP-2, resulting in proMMP-2 activation and release [13,14]. While TIMP-2 levels were reported to be significantly elevated in ovarian clear cell carcinoma [21], the expression of MMP-14 has not been evaluated. In addition to functioning as a proMMP-2 activator, recent studies have demonstrated that MMP-14-catalyzed pericellular collagenolysis promotes tissue invasion of a number of tumor cells [15,22-25] and enables cells to defeat growth regulatory checkpoints, thereby enabling proliferation in an otherwise constrained three dimensional collagen or fibrin microenvironment [26].

In the current study, ovarian clear cell tumors were evaluated using immunohistochemistry for expression of MMP-14, MMP-2 and MMP-9. Expression levels were compared to those in other histotypes (serous, endometrioid, mucinous). Complementary *in vitro* analyses examined MMP-14 expression and function in the clear cell ovarian carcinoma cell line ES2.

Materials and Methods

Case Selection and Tissue Microarray

A total of 163 cases of primary epithelial ovarian tumors from oophorectomies performed at Northwestern Memorial Hospital during the period of 1999 to 2003 were collected with Institutional Review Board-approved consent from the Surgical Pathology archives. Paraffin-embedded donor tissue blocks were sampled with 1.5 mm punchers using a Beecher tissue microarray (TMA) instrument (Beecher Instruments Inc, Sun Prairie, WI). Gross and histological diagnoses were confirmed in all cases by two pathologists (B. P. A. or X. J. Y.). Two TMAs containing a total of 71 serous carcinomas, 45 endometrioid carcinomas, 18 clear cell carcinomas, and 9 mucinous tumors were constructed. Note that in specific serial sections,

1-2 cores may not contain an adequate amount of tumor for analysis, resulting in slight variations in sample size.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μ m sections obtained from formalin-fixed, paraffin-embedded tissue microarray blocks. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Antigen retrieval was carried out in citrate buffer (10mM, pH-6) for 15 minutes at 100°C in a microwave oven. Monoclonal mouse anti-human MMP-2, and polyclonal rabbit anti-human MMP-9 and MMP-14 (NeoMarkers, Inc., Fremont, CA) antibodies at 1:50 were applied for 30 minutes at room temperature. Subsequent reactions were performed with biotin-free HRP enzyme labeled polymer of EnVision plus detection system (DakoCytomation, Carpinteria, CA). Positive reactions were visualized with diaminobenzidine solution followed by counterstaining with hematoxylin. Appropriate negative controls for the immunostaining were prepared by using non-immune mouse IgG.

Evaluation of Immunohistochemistry

Immunoreactivity was evaluated in a semi-quantitative manner by scoring intensity on a 0 to 3 scale. Positive staining for MMP-2, MMP-9, and MMP-14 was defined as coarse, dense cytoplasmic and/or membranous granularity. Staining intensity was divided into four categories: 0=negative, 1=weak, 2=moderate and 3=strong. To reduce interobserver variation in the evaluation of staining patterns, the above semi-quantitative method was used to independently score immunohistochemical staining of the tumors by two pathologists (B.P.A and X.J.Y.) and a consensus intensity score was achieved.

Materials and Cell Lines

The broad spectrum metalloproteinase inhibitor GM6001 was purchased from Chemicon (Temecula, CA). Lysophosphatidic acid (LPA 18:1) was from Avanti (Alabaster, AL). Type 1 collagen from rat tail and transwell inserts were obtained from Becton-Dickinson (Bedford, MA). Sulfo-NHS-biotin analog [sulfo-succinimidyl-6- (biotinamido) hexanoate] and NeutrAvidin beads were from Pierce (Rockford, IL). Concanavalin A and all other reagents were purchased from Sigma (St. Louis, MO). The ES2 clear cell ovarian cancer cell line was purchased from American Type Culture Collection (Manassas, VA). DOV13 serous adenocarcinoma cell line was a generous gift from Dr. Robert Bast Jr., (M.D. Anderson Cancer Center, Houston, TX) and was used as a control cell line that expresses high levels of endogenous MMP-14 [15,24]. Cells were routinely maintained in MEM supplemented with 10% fetal bovine serum, 1mM essential amino acids, 1mM sodium pyruvate, and 50U/ml penicillin and streptomycin under standard conditions. In addition, DOV13 growth media was supplemented with 0.11U/ml insulin. Cells were plated in 6-well tissue culture dishes at 70-80% confluency. Cells were serum starved for three hours before addition of 25 μ M GM6001, 20mg/ml concanavalin A, or 80 μ M LPA [27] and analyzed as described below.

MMP-14 Immunoblots and Labeling of Cell Surface Proteins

Cells were incubated in serum-free media and treatments for 24 hours. Cell lysates were electrophoresed on 9% SDS-polyacrylamide gels under reducing conditions followed by transfer to PVDF membrane as described previously [28]. Membranes were incubated overnight at 4°C with 1:1000 dilution of anti-MT1-MMP hinge antibody (Sigma) followed by peroxidase-conjugated anti-(mouse-IgG) antibody (1:5000). Immunoreactive bands were visualized using enhanced chemiluminescence substrate (Pierce, Rockford, IL). Isolation of biotin-labeled cell surface proteins was conducted as previously described [28]. Briefly, cells were washed twice with PBS. Cell surface proteins were labeled with a non-cell permeable sulfo-NHS biotin analog. Sulfo-NHS-biotin (1mg/ml in PBS containing calcium and

magnesium) was added to each well of 6-well plate and incubated on ice for 30 minutes with gentle shaking. Cells were then washed three times with cold 100mM glycine in PBS containing calcium and magnesium, followed by one wash in PBS. Cells were lysed and protein concentrations were determined using Bio-Rad D_c detection kit (Hercules, CA). Equal amounts of protein in lysis buffer were added to 1.5 ml tubes and incubated with 40µl NeutrAvidin coated beads on rotary wheel at 4°C for 12 hours. Beads were washed five times with lysis buffer before bound proteins were denatured by boiling, separated by SDS-PAGE, immunoblotted, and probed for MT1-MMP as described above.

Gelatin Zymography

Gelatinase activity of ES2 and DOV13 conditioned media (20µl) was measured using 9% SDS-polyacrylamide gels containing 0.1% gelatin as described previously [27]. Gels were washed in 2.5% Triton X-100 for 30 minutes prior to incubation in 20mM Glycine, 10mM CaCl₂, 1µM ZnCl₂ (pH 8.3) for 48 hours at 37°C, before staining with Commassie Brilliant Blue.

Transwell Invasion Assay

Invasion assays were conducted as previously described [23,27]. Human type I collagen (Sigma) was prepared as a stock solution by dissolving in 0.5M acetic acid to a concentration of 2mg/ml. Transwell inserts were coated on underside with 500µl collagen stock diluted to 100µg/ml in 100mM Na₂CO₃ for 1hr at 37°C. Inserts were allowed to dry at room temperature overnight and washed three times with PBS. Cells (75,000) in serum-free media (500µl) were added to the inner portion of each well. Serum-free media (750µl) was added to outer chamber and plates were incubated for 20 hours. Inner chambers were cleaned using a cotton swab and invaded cells were fixed and stained using Diff-Quik staining solutions (Dade Behring, Newark, DE). Invaded cells were quantified by counting 10 high powered fields per invasion chamber and averaging the number of cells per randomly chosen high power field. Experiments were performed in triplicate and were repeated at least two times.

Three-Dimensional Growth Assay

Type I collagen from rat tail, solubilized in 0.5M acetic acid (Becton-Dickinson, Bedford, MA) was neutralized with 0.1N sodium hydroxide, and diluted in 10X PBS to 4mg/ml. This solution was then mixed 1:1 with growth media containing 1×10^3 cells/ml and 600µl of the 2mg/ml solution was added to each well of a 24-well tissue culture dish [26]. The solution was incubated at 37°C for 30 minutes to allow the gel to solidify. Growth media (500µl) was then added to each well. Individual cells were photographed at the time of plating and after 5 days of incubation.

Results

MMP Immunohistochemical Evaluation

To assess the potential contribution of MMP-14 and MMP-2 to ovarian clear cell carcinomas, immunohistochemical staining was evaluated by histotype, with the related secreted MMP-9 included as a control. Ninety-five percent of clear cell carcinomas (17/18) expressed moderate to high (2+ - 3+) levels of MMP-14 (Table 1, Fig. 1) compared with 45% (32/70) of serous carcinomas, 52% (23/44) of endometrioid carcinomas, and 55% (5/9) of mucinous carcinomas (Table 1). Conversely, only one clear cell carcinoma was negative for MMP-14 compared to 27 of 142 (20%) of non-clear cell tumors. Similarly, ninety-four percent of clear cell carcinomas (16/17) expressed moderate to high levels (2+ - 3+) of MMP-2 compared with 55% (39/71) of serous carcinomas, 49% (22/45) of endometrioid carcinomas, and 33% (3/9) of mucinous carcinomas (Table 1, Fig. 1). Conversely, only one clear cell carcinoma was negative for MMP-2 compared to 46 of 145 (35%) of non-clear cell tumors. Of note, the same clear cell

carcinoma was negative for both MMP-2 and MMP-14. This tumor was also negative for MMP-9. As a control, expression of MMP-9, a related secreted MMP, was evaluated. MMP-9 was expressed at moderate to high levels (2+ - 3+) in only 11% of clear cell carcinomas (2/18), but was more widely expressed in other histotypes: 28% (20/71) of serous carcinomas, 22% (10/45) of endometrioid carcinomas, and 22% (2/9) of mucinous carcinomas (Table 1).

MMP-14 Expression in Clear Cell Ovarian Carcinoma *in vitro*

As the immunohistochemical data demonstrated strong expression of MMP-14 in human tumors, the clear cell ovarian cancer cell line ES2 was evaluated to explore further the role of MMP-14 *in vitro*. As a positive control, expression levels were compared to the well-characterized ovarian serous adenocarcinoma cell line DOV13, previously demonstrated to express very high levels of surface-active endogenous MMP-14 [24]. Note that MMP-14 expression in cell lines derived from ovarian serous adenocarcinoma is heterogeneous, as seen in ovarian carcinomas (Table 1), and other serous adenocarcinoma-derived cell lines such as OvCa429 and OvCa433 do not express detectable endogenous MT1-MMP protein (data not shown). Total MMP-14 expression was determined by western blotting of whole cell lysates, while a surface labeling/affinity precipitation protocol was used to evaluate cell surface MMP-14 levels. Both the active 55 kDa MMP-14 species and the 43 kDa autolysis product were found in whole cell lysates (Fig. 2A, arrow and arrowhead, respectively), while only the 55 kDa species was detected on the surface of ES2 cells (Fig. 2B, arrow).

Because MMP-14 functions as a physiologic activator of proMMP-2, gelatin zymography was used to evaluate activated MMP-2 in the conditioned media of ES2 cells. ES2 cells expressed levels of proMMP-2 comparable to the DOV13 control and were induced to activate proMMP-2 following treatment with concanavalin A, but not LPA [27] (Fig. 3A). Ovarian cancer cells have been shown to respond to three dimensional (3D) collagen culture by upregulation of MMP-14 expression and/or activity [15,24,29]. To evaluate the effect of 3D collagen culture on MMP-14 activity, ES2 cells were cultured for 24 hours in 3D collagen in the presence or absence of ConA. Although the response of ES2 cells to ConA is more robust in 3D cultures (Fig. 3B), three-dimensional collagen did not appear to be a major regulator of MMP-14 expression or activity in ES2 cells.

To determine the overall contribution of MMPs to cellular invasive potential, transwell invasion chambers coated with a thin layer of collagen were utilized. Cells were plated for 20 hours in the collagen-coated transwells in the presence or absence of a general MMP inhibitor, GM6001. Similar to DOV13 cells, ES2 cells invaded the collagen-coated transwell, and this activity was blocked by the broad spectrum MMP inhibitor GM6001 (Fig. 4). In addition to its role in potentiating tissue invasion [22-25], MMP-14 has also been reported to impart a growth advantage to cells in a three-dimensional microenvironment [26], such as is encountered during tumor growth in a provisional fibrin matrix or to the secondary organs of the peritoneal cavity. To model these events, ES2 cells were plated within three-dimensional type I collagen gels, as the submesothelial matrix of the peritoneal cavity is comprised predominantly of interstitial collagens and ovarian tumors induce a fibroproliferative response characterized by enhanced peritoneal type I collagen synthesis [30-32]. Cells in three-dimensional culture are completely encompassed within the matrix and encounter collagen in all directions. Furthermore, cells in the three-dimensional matrix are plated at single-cell density and must therefore participate in extensive degradation and remodeling of the surrounding collagen matrix during expansion of the cell population without paracrine signals from neighboring cells. ES2 cells survived, proliferated and migrated within three-dimensional collagen gels over a 5 day time period (Fig. 5).

Discussion

Clear cell carcinomas of the ovary comprise approximately 5% of all ovarian neoplasms and display a distinct gene expression profile relative to other histotypes [2,5,6]. Ovarian clear cell carcinomas exhibit unique features including a more aggressive clinical course and more malignant behavior relative to stage matched serous adenocarcinomas [7,9,11,12]. The current immunohistochemical analysis of a panel of ovarian tumors demonstrates markedly strong expression of MMP-14 in 95% of clear cell tumors, relative to approximately 45-55% expression levels in serous, endometrioid and mucinous tumors. MMP-14 functions as both a pericellular collagenase [33] and as an activator of proMMP-2 via formation of a MMP-14/proMMP-2/TIMP-2 complex [13,14]. MMP-2 was also strongly expressed by clear cell carcinomas, with 94% showing strong positive immunoreactivity. This is in agreement with a previous study that used *in situ* zymography to demonstrate that clear cell carcinomas express strong gelatinolytic activity [34]. Interestingly, expression of TIMP-2, a member of the cell surface trimolecular zymogen activation complex along with MMP-14 and MMP-2, has also recently been shown to be elevated in the epithelial compartment of ovarian clear cell tumors [21], supporting the current results.

Although outcome data are not available for the patients whose tumors were evaluated in the current study, several published studies have examined the potential prognostic significance of MMP-2 and MMP-14 expression in epithelial ovarian cancers; however the histotype-specificities of these findings were not reported. These analyses are further complicated by the finding of both epithelial and stromal MMP expression, although immunohistochemical staining in the current study revealed staining largely associated with the epithelial compartment. A number of studies have reported that high epithelial and/or stromal expression of MMP-2 is associated with poor survival [36-39]; however high epithelial MMP-2 expression has also been correlated with better disease-related survival [40]. It has been speculated that MMP-2 may contribute to tumor cell invasion regardless of the source of the proteinase [39], and this is supported by *in situ* hybridization studies demonstrating that stromal MMP-2 signals are localized at the tumor-stroma interface in desmoplastic fibroblasts [18]. MMP-14 has also been localized to both stromal and epithelial cells [39], although *in situ* hybridization analyses detected MMP-14 exclusively in tumor cells [18]. High expression of epithelial MMP-14 correlated with the development of metastases, poor prognosis, and the shortest disease-specific survival [39].

Based on the immunohistochemical data, expression and function of MMP-14 was evaluated using the single ovarian clear cell carcinoma cell line ES2. MMP-14 was expressed, localized to the cell surface, and active in catalyzing proMMP-2 activation. Furthermore, ES2 cells invaded collagen gels and exhibited extensive proliferation in three-dimensional collagen. In addition to activating proMMP-2, MMP-14 is also a potent interstitial collagenase [33] and this activity may potentiate anchoring and dissemination of metastatic ovarian tumors in the interstitial collagen-rich peritoneal microenvironment [15,24,27,29,35]. Clear cell tumors tend to present at earlier stage than serous adenocarcinoma and this is thought to result from the frequent presentation of these tumors as a large pelvic mass [7,11,12]. The reason for advantageous intraperitoneal growth and enhanced metastatic dissemination of clear cell carcinomas is unclear, but has been attributed in part to enhanced chemoresistance and dysregulated growth control [2,5,6,11]. Many studies have linked MMP-14 activity to invasion in multiple tumor types [14,41,42]. However, MMP-14 has also been shown to confer a three-dimensional growth advantage to tumor cells, enabling cells to remove matrix constraints and thereby drive a proliferative response in an otherwise physically constrained environment [26]. Our dataset contained three stage I clear cell carcinomas, all of which scored strong positive for MMP-14. Taken together, it is interesting to speculate that MMP-14 may contribute both to growth of the primary tumor mass as well as dissemination of ovarian clear cell tumors.

As clear cell tumors are generally insensitive to conventional platinum-based chemotherapy [9], these data suggest that a combination therapy that includes an MMP inhibitor may provide a novel strategy for treatment of clear cells carcinoma of the ovary.

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Figure 1. Immunohistochemical analysis of serial human clear cell ovarian tumor sections for MMP-14 and MMP-2

Serial sections of primary ovarian tumor samples were stained with (A) H&E or antibodies to (B) MMP-2 or (C-F) MMP-14 (as indicated) and scored as described in “Materials and Methods”. Strong positive staining for both MMP-14 and MMP-2 was observed in 94% (17/18) of tumors analyzed and examination of serial tumor sections revealed co-localization of staining. Panels (D-F) show examples of tumors with staining intensity scored as weak (1+), moderate (2+) and strong (3+), respectively. 400X magnification.

Figure 2. MMP-14 protein expression in ES2 cells

Cells were grown to confluence, serum-starved for 3 hours and incubated in the presence or absence of concanavalin A (20 ug/ml) for 24 hr in serum-free media. (A) Whole cell lysates were probed for MMP-14 protein using anti-MT1-MMP hinge Ab (1:1000 dilution) and HRP-conjugated secondary antibody (1:5000 dilution) followed by ECL detection. (B) Cell surface expression of MMP-14 was determined by labeling cell surface proteins with a non-cell permeable biotin analog followed by streptavidin precipitation and probing the labeled protein pool for MMP-14 as described above. In control experiments, DOV13 cells were used for comparison.

Fig. 3A

Fig. 3B

Figure 3. MMP-14-catalyzed proMMP-2 activation

(A) Cells were grown to confluence, serum-starved for 3 hours and incubated in the presence or absence of concanavalin A (20 μ g/ml) or LPA (80 μ M) for 24hr in serum-free media. Gelatinase activity of conditioned media was detected using zymography. The migration positions of pro- and active MMP-2 are indicated. (B) Effect of three dimensional collagen culture on MMP-14 activity. Cells were grown to 70-80% confluence in 6-well culture plate. Serum-free media (1 ml) or type I collagen (0.8 mg/ml) diluted in serum-free media, containing concanavalin A (20 μ g/ml) or vehicle, was added to each well. After 48 hours incubation, conditioned media or collagen gel was collected and centrifuged. Presence of active MMP-2 in conditioned media was determined using gelatin zymography. Control samples contained the broad spectrum MMP inhibitor GM6001 (25 uM, designated GM).

Figure 4. Invasion of type I collagen gels by ES2 cells

Cells were grown to confluence and serum starved for 3 hours before trypsinization. Equal numbers of cells in serum-free media were added to the inner chamber of collagen-coated inserts (100 μ g) and incubated for 20 hours. Invading cells were fixed, stained, and counted. Results are the average of two experiments of triplicate wells. Error bars indicate standard deviation.

Figure 5. ES2 cells demonstrate a growth advantage in 3D collagen

Cells (1×10^3) were plated in neutralized collagen (500 μ l) at a concentration of 2mg/ml. Collagen mixture was incubated at 37°C for 30 minutes to allow gel to form. Growth media was added to each well. Cells within collagen gel were photographed using phase-contrast microscopy 1 and 5 days after plating.

Table 1
Immunohistochemical Analysis of MMP Expression in Epithelial Ovarian Tumors

Microarrayed human ovarian carcinoma tissues were subjected to immunohistochemical staining for matrix metalloproteinases-2 and -9 (MMP-2, MMP-9) or membrane type 1 MMP (MMP-14) as described in Materials and Methods. Semi-quantitative analysis of staining was performed by two pathologists (BPA and XJY) and scored as negative (0), weak (1), moderate (2) or strong (3). Statistical analysis of the correlation between MMP and clear cell carcinoma histotype was performed using Fisher's exact test. MMP-2: p<.001; MMP-9: p=.71; MMP-14: p=.04.

Tumor type:	MMP-2				MMP-9				MMP-14			
	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
clear cell	1/17 6%	0/17 0%	3/17 18%	13/17 76%	4/18 22%	12/18 67%	2/18 11%	0/18 0%	1/18 6%	0/18 0%	14/18 78%	3/18 17%
serous	26/71 37%	6/71 8%	8/71 11%	31/71 44%	16/71 23%	35/71 49%	17/71 24%	3/71 4%	14/70 20%	24/70 34%	24/70 34%	8/70 11%
endometrioid	15/45 33%	8/45 18%	13/45 29%	9/45 20%	14/45 31%	21/45 47%	9/45 20%	1/45 2%	11/44 25%	10/44 22%	19/44 43%	4/44 9%
mucinous	5/9 56%	1/9 11%	3/9 33%	0/9 0%	3/9 33%	4/9 44%	1/9 11%	1/9 11%	2/9 22%	2/9 22%	3/9 33%	2/9 22%