

OC125, M11 and OV197 epitopes are not uniformly distributed in the tandem-repeat region of CA125 and require the entire SEA domain

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Abstract. The human cancer antigen 125 (CA125) is over-expressed in epithelial ovarian cancer cells and it plays a role in the pathogenesis of ovarian cancer. This protein presents a repeat region containing up to sixty tandem repeat units. The anti-CA125 monoclonal antibodies have been previously classified into three groups: two major families, the OC125-like antibodies and M11-like antibodies, and a third group, the OV197-like antibodies. A model in which a single repeat unit contains all the epitopes for these antibodies has been also proposed, even if their exact position is still undetermined. In the present work, the affinities of the monoclonal antibodies, representative of the three families, have been investigated for different CA125-recombinant repeats through Western blot analysis. Different patterns of antibody recognition for the recombinant repeats show that CA125 epitopes are not uniformly distributed in the tandem repeat region of the protein. The minimal region for the recognition of these antibodies has been also individuated in the SEA domain through the subcloning of deleted sequences of the highly recognized repeat-25 (R-25), their expression as recombinant fragments in *E. coli* and Western blot analysis. Obtained data have been further confirmed by ELISA using the entire R-25 as coating antigen.

Keywords: CA125, ovarian carcinoma, epitope type, SEA domain, monoclonal antibodies

1. Introduction

The human cancer antigen 125 (CA125) is a membrane-associated mucin-type glycoprotein [16,17], encoded by the *MUC16* gene. It was identified through the OC125 monoclonal antibody (mAb) that bound to the surface of the ovarian cancer cells OVCA 433 [27]. It is normally found in abdominal fluid and in cervical mucus [10,15], in female reproductive organs, in pleura, pericardium and peritoneum, in the epithelium of the ocular surface and the in the upper respiratory tract [1,11,23,38].

A remarkable aspect of CA125 is its over-expression in epithelial ovarian cancer cells as well as in other carcinomas [6,9,18]. The CA125 aberrant expression might play a role in the pathogenesis of ovarian cancer. It has been suggested that CA125 might protect the ovarian cancer cells from NK immune surveillance [22] and from the attack of complement factors [36]. Since CA125 interacts with mesothelin, a protein expressed by the mesothelial cells of peritoneum, it might promote the metastatic dissemination of ovarian cancer cells [13]. Besides, CA125 directly regulated cell growth, tumorigenesis and metastasis of ovarian cancer cells in *in vitro* experiments [5,29].

CA125 is released from the surface of the cells into the blood and it is used as ovarian tumoral marker in serum assays [25]. Although the CA125 detection

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shows low specificity and high rates of false positives in early disease, it represents a useful tool to monitor the progression or the regression of ovarian carcinomas after surgery and cytotoxic therapy [25,26].

The antigenic properties of CA125 are also considered for therapeutic approaches against ovarian cancer. Indeed, anti-CA125 mAbs were evaluated as radionuclide or cytotoxin conjugated [20,37], as anti-idiotypic antibody in order to mime the OC125 epitope and to elicit an antitumor immune response [12,19], or as immunoglobulins to induce an antibody-dependent cellular cytotoxicity [3].

Many CA125 features may be elucidated by its molecular structure. The cloning of the human MUC16 gene provided some indication on the structure of the encoded protein [33,34]. It is up to 22,152 amino acid (aa) long, with a molecular weight of 2.5 MDa that has to be doubled considering glycosylation. The protein consists of a highly glycosylated N-terminal extracellular region, a multiple repeat domain, containing up to sixty tandem repeat units, a carboxy terminal domain, presenting a potential proteolytic cleavage site proximal to the membrane, a transmembrane anchor and a short cytoplasmic tail [34]. It should be considered that CA125 may show biochemical and molecular heterogeneity depending on the biological sources, the use of different isolation techniques and different isoforms [8]. A single repeat unit is about 156 aa long [4, 34] and contains a SEA module, a module found in sea urchin sperm protein, enterokinase and agrin [24], which presents a sandwich three-dimensional structure formed by two alpha helices, four antiparallel beta strands and a hydrophobic core [24,32]. Several hypotheses for the SEA module function were proposed, like protein recognition, nucleic acid as well as sugar chain binding activity, interaction with matrix molecules [24,32].

All the antigenic domains of CA125 have been reported in the repeat region [14]. Indeed, twenty-six anti-CA125 mAbs defined only three antigenic domains, that were classified into three families [14]: two major groups, the OC125-like antibodies (group A) and the M11-like antibodies (group B), and a third group C represented by the OV197 antibody. At present, the exact position of CA125 epitopes in the repeat units remains undefined even if a model in which a single repeat unit encompasses all the three antigenic domains has been proposed [7,34].

The heterologous expression of one of the CA125 repeats, the repeat 11 (R-11: the numbering of the repeats starts from the N-terminus of CA125 according

to O'Brien et al. [34]), highlighted that the OC125 mAb poorly reacted with this unit both in antigen capture and cross-inhibition methods and that the prior binding of some mAbs from the group B enhanced subsequent OC125 recognition. It has been argued that CA125 epitopes need to be contained in the CA125 tandem repeat region to be optimally displayed and that they may be conformationally dependent [7,14].

Although the amino acid sequences of CA125 repeats are highly homologous, they are not perfectly conserved [33,34]. We investigated the affinity of mAbs, representative of the CA125 antigenic domains, for several CA125 recombinant repeats in addition to R-11.

For the first time, the cloning and the heterologous expression of different repeat units of CA125, including the R-11, is here reported. The OC125 and the OV197 mAbs recognized the recombinant repeats with very different efficiencies. The M11 mAb recognized all the recombinant repeats, although with some differences. For high bindings to the recombinant repeats, incubations with single antibodies were sufficient, both in immunoblot and in ELISA analysis. A deletion analysis showed that the region of the R-25 containing the entire SEA domain was necessary to be recognized by CA125 mAbs.

These data indicate that the affinity of mAbs for the CA125 repeats dramatically depends on the primary structures of their amino acid sequences and confirm the conformational type of CA125 epitopes.

2. Materials and methods

2.1. Materials

Human ovarian tumor epithelial OAW28 cells were from ECACC, Salisbury, UK. They were routinely cultured in DMEM complemented with 10% FBS, 2 mM Glutamine, 20 IU/I Bovine Insulin and 1 mM Sodium Pyruvate (Invitrogen, Paisley, UK).

For the cloning of the CA125-repeat units, amplification primers were synthesized by Primm (Milan, Italy). The DNA polymerase enzyme used was the PfuUltra[®] Hotstart DNA polymerase from Stratagene (La Jolla, CA, USA). The restriction enzymes were purchased from Roche Applied Science (Mannheim, Germany). The competent cells used for bacterial transformations derived from the JM109 *E. coli* strain and were purchased from Stratagene.

Western blots were performed with the anti-CA125 mouse mAb (OC125) (Calbiochem Merck, Darmstadt,

Germany), the monoclonal mouse anti-human CA125 clone M11 (DakoCytomation, Carpinteria, CA, USA), the OV197 (Fujirebio Diagnostic, Göterborg, Sweden), the secondary antibody goat anti-mouse IgG (H+L)-HRP Conjugate (Bio-Rad, Hercules, CA, USA) and with the penta-His HRP conjugate antibody (Qiagen, Valencia, CA, USA).

ELISA on the purified His-tagged hCA125-repeat units were performed using Ni-NTA HisSorb 96-well plates from Qiagen, the mentioned primary antibodies and the peroxidase-conjugated affiniPure goat Anti-Mouse IgG, Fc γ Fragment Specific as secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). For the ELISA of the His-tagged murine DHFR, the primary antibody was a rabbit polyclonal anti-DHFR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), while the secondary antibody was the peroxidase-conjugated affinipure goat anti-rabbit IgG, Fc fragment specific from Jackson ImmunoResearch.

2.2. Methods

2.2.1. Cloning of His-tagged hCA125-repeat units in *E. coli*

The cloning of CA125-repeat units was executed with QIAexpress Type IV Kit (Qiagen). This kit contains the expression vector pQE-30, which is used for the expression of 6 \times His-tagged recombinant proteins, and the expression vector pQE-40 that expresses a His-tagged murine DHFR protein and is used as control or for chimeric constructs. His-tagged proteins may be purified through nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography and used as antigen in ELISA with Ni-NTA coated plates, according to the instructions of the kit manual.

Total RNA was isolated and purified from OAW28 cells using the SV Total RNA Isolation System from Promega (Madison, WI, USA); RNA yield and the integrity were determined spectrophotometrically and by gel electrophoresis respectively. The cDNA was synthesized with the Reverse Transcription System (Promega) and used in PCR for the amplification of CA125 tandem repeat units. For the cloning of the R-11 unit, R11-U and R11-L primers were synthesized, according to the oligo sequences published by T J O'Brien et al. [34].

A second repeat cloning was performed with the forward primer U-I and the reverse primer L-X (Table 1). PCR reaction mixtures were set up with cDNA deriving from 300 ng of total RNA, 25 pmol of each primer, 50 nmol MgCl₂, 50 nmol dNTP, buffer 1 \times and

2.5 U Pfu, in a final volume of 50 μ l. The PCR amplification protocol for R-11 was 5 min at 95°C, for the initial denaturation and enzyme activation, 7 cycles consisting of denaturation at 95°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 2 min, 25 cycles at 95°C for 1 min and 72°C for 3 min, and a final step at 72°C for 7 min. The amplification with the second primer pair was performed at an initial annealing of 63°C, instead of the calculated 65°C, to amplify more different repeat units in sub-stringent conditions. Amplifications took place in a 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The amplified products were checked by gel electrophoresis and purified with the PCR cleanup Gel extraction kit (Macherey-Nagel, Duren, Germany). Both the expression vector pQE-30 and the purified inserts were digested with Bam HI and Hind III restriction enzymes. The digestion products were separated by electrophoresis. The DNA band corresponding to the digested vector was excised and purified with the described kit, while the digested inserts were run and excised from the low melting gel NuSieve GTG (Lonza, Walkersville, MD, USA), and directly used as melted gel in the ligase reaction. The reaction of ligation between the expression vector and insert was performed with the Rapid DNA ligation kit (Roche Applied Science). Ligation mixtures were used to transform JM109 *E. coli* competent cells. Bacterial clones were grown and plasmidic DNA was prepared with Nucleobond Xtra Maxi Plus and Nucleospin Plasmid kits from Macherey-Nagel. Plasmidic DNAs were characterized by restriction analysis.

2.2.2. Subcloning of His-tagged deleted regions of the R-25 unit in *E. coli*

The subcloning of nucleotidic sequences encoding R-25 subregions, both in pQE-30 and in pQE-40 expression vectors, was performed as described above for the cloning of the entire repeat in pQE-30. Ten nanograms of pQE30-R25, the vector containing the entire cloned R-25 sequence, were used as template in PCR reactions. Amplification primers, besides the U-I and L-X primers described as above, were: the upper primers U-II, U-III, the lower primers L-VIII and L-IX (Table 1). Schematic representations of all the products encoded by the obtained clones are reported in Fig. 3A, for the pQE-30 subclones, and in Fig. 4A, for the chimeric products with murine DHFR.

2.2.3. Sequencing

The nucleotidic sequences of inserts, as well as the correct in frame ligation, were determined through nucleotidic sequencing by Primm (Milan, Italy).

Table 1
Primers used for the amplification and cloning of CA125 repeats and the R-25 regions

| Primer name: | Primer sequence (5'-3') | Amplified CA125 regions |
|--------------|--|-------------------------|
| R11-U: | <u>ACCGGATCCGGCCACACAGAGCCTGGCCC</u> | R-11 |
| R11-L: | TGT <u>AAGCTT</u> AGGCAGGGAGGATGGAGTCC | |
| U-I: | <u>ACCGGATCCAGCCCCACA</u> ACTGCTGGCCCT | R-2, -7, -9, -25, -51 |
| L-X: | TGT <u>AAGCTT</u> GGGGAGGGAGGATGGAGTCCCTGA | |
| U-II: | <u>CGGGGATCCGGCTCCAGGAAGTTCAACACCACA</u> | R-25 regions |
| U-III: | <u>CGGGGATCCGGTCCC</u> ATGTTCAAGAACCACAGT | |
| L-VIII: | CCG <u>AAGCTT</u> GTATAGCTGCTCCCTGTCCACT | |
| L-IX: | CGG <u>AAGCTT</u> GGTAAACCATTGACATAGAGACT | |

Restriction sites are underlined. GGATCC: Bam HI, AAGCTT: Hind III. U: upper primer, L: lower primer.

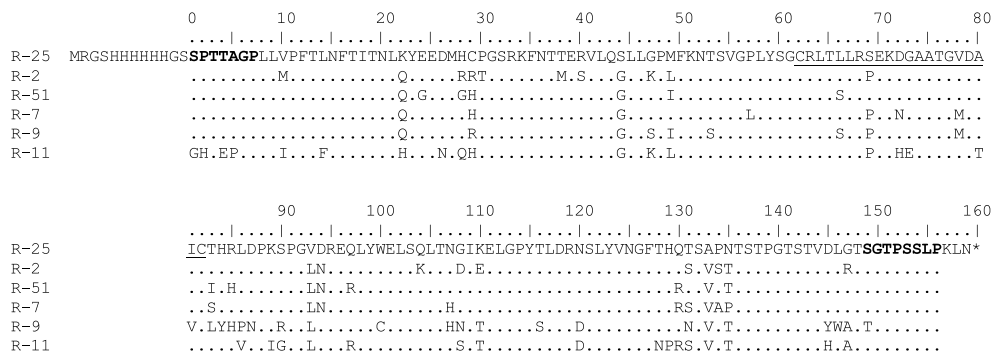


Fig. 1. Alignment of the amino acid sequences of six recombinant CA125 repeats subcloned and expressed in *E. coli*. Not aligned residues at the amino- and carboxy- ends are encoded by plasmidic sequences. The SEA domain extends from position 0 to 126 [32], while the cysteine loop region is underlined. Residues in bold are shared by the R-25, -2, -51, -7 and -9, since they all derived from the primers used for the amplification of the R-25 encoding sequence (see Table 1).

2.2.4. Expression of $6 \times$ His-tagged hCA125-repeat units in *E. coli* and Western blot analysis

According to the QIAexpressionist handbook (Qiagen), the obtained bacterial clones were grown at 37°C to $OD_{600} = 0.5$ and induced for 4 h with 1mM IPTG to produce the $6 \times$ His-tagged hCA125-repeat units. Bacterial pellets were lysed with Buffer B (100 mM NaH_2PO_4 , 10 mM Tris Cl, 8 mM Urea, pH 8). NuPAGE® LDS Sample buffer with reducing agent was added to lysates that were run on NuPAGE® 4–12% Bis-Tris gel (Invitrogen, Grand Island, NY, USA) at 200 V for 30 min. Proteins were electroblotted at 30 V 1h on 0.2 μ m PVDF membrane (Bio-Rad) and the transfer was controlled with Red-Ponceau staining (Sigma, St. Louis, MO, USA). Membranes were incubated 1h at room temperature (RT) in blocking buffer (5% non-fat milk, 0.05% Tween-20, 1 \times PBS or in casein 1%). The primary antibodies were incubated in the same buffer and at RT for 2 h. Depending on the immuno-detection, the primary antibodies were 2.5 μ g/ml anti-CA125 mouse mAb (OC125) or 2.5 μ g/ml monoclonal mouse anti-human CA125 clone M11 or 2.5 μ g/ml OV197 mAb. After three washes with wash buffer (0.1% Tween-20, 1 \times PBS)

at RT for 10 min, membrane were incubated with 1:3000 goat anti-mouse IgG (H+L)-HRP conjugate at RT for 1h. After four washes, chemiluminescent detection was performed with the ECL Western Blotting Detection Reagents on X-ray film (Amersham GE Healthcare, Little Chalfont, UK). When 0.1 μ g/ml Penta-His Antibody (Qiagen) was used as primary antibody, the blocking and incubation buffer was 1 \times PBS/Casein blocker (Bio-Rad). For reprobing with different antibodies, membranes were before incubated in stripping buffer (20 ml SDS 10%, 12.5 ml Tris HCl pH 6.8, 0.5 M, 0.8 ml betamercaptoethanol) at 50°C for 45 min.

2.3. Expression and purification of the R-25

Protein expression and purification was performed following the handbook of the QIAexpress Type IV Kit (Qiagen). Briefly, both a selected bacterial clone expressing hCA125-repeat unit and the one expressing murine DHFR as control protein (Qiagen) were grown at $OD_{600} = 0.5$ and induced for 5 h with 1 mM IPTG. Each bacterial pellet, derived from 0.5 L of culture,

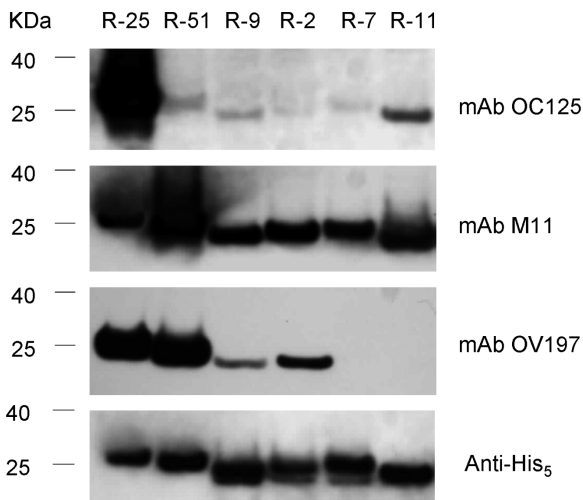


Fig. 2. Western blot analysis of six recombinant CA125 repeats. *E. coli* extracts containing the indicated recombinant repeats were probed with the monoclonal antibodies OC125, M11, OV197 and with the anti-penta histidine as control antibody. The numbering of the repeats starts from the N-terminus of CA125 according to O'Brien et al. [34].

was lysed through incubation with 100 ml buffer B (see above) at RT for 1 h. After $10000 \times g$ centrifugation at RT for 30 min, each supernatant was incubated with 1–1.5 ml of 50% Ni-NTA resin (Qiagen) at RT for 1 h, on a rotary shaker at 200 rpm. The supernatant-resin mixtures were loaded into empty columns. The columns were washed 3–4 times with 5 ml buffer B, 5–7 times with 5 ml buffer C (100 mM NaH_2PO_4 , 10 mM Tris Cl, 8 mM Urea, pH 6.3), and proteins were eluted 4–8 times with 0.5 ml buffer D for protein monomers (100 mM NaH_2PO_4 , 10 mM Tris Cl, 8 mM Urea, pH 5.9) followed by 4–8 times with 0.5 ml buffer E for protein multimers (100 mM NaH_2PO_4 , 10 mM Tris Cl, 8 mM Urea, pH 4.5). Eluted proteins were controlled on 10–20% SDS PAGE gels stained with Bio-Safe Coomassie G-250 (Bio-Rad). The elution fractions containing the most of the proteins were pooled and the resulting protein concentration was assessed with the Protein Assay of Bio-Rad.

2.3.1. Immunodetection of the hCA125 R25 by ELISA

The nickel-nitrilotriacetic acid (Ni-NTA) is also used in the Ni-NTA HisSorb 96-well plates to capture tagged protein from cleared lysates as well as to perform ELISA. ELISA on the purified His-tagged hCA125-repeat unit and His-tagged murine DHFR control were performed following the instruction of the QIAexpress Detection and Assay Handbook (Qiagen) with some modifications. Briefly, for the coating

of the plates, 250 ng of $6 \times \text{His-hCA125}$ -repeat unit, in 200 μl of blocking buffer (0.2% BSA, PBS $1 \times$, pH 7.4), were added to each well and incubated at RT for 1 h. After four washes with 300 μl PBS-Tween (0.05% Tween-20 in PBS $1 \times$), 200 μl /well of 1 $\mu\text{g}/\text{ml}$ mAbs in blocking buffer were added and incubated at RT for 2 hours. After four washes with 300 μl PBS-Tween, 200 μl /well of secondary antibody in blocking buffer (1:20000 Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG, $\text{Fc}\gamma$ Fragment Specific) were added and incubated at RT for 1 hour. After four washes with 300 μl PBS-Tween, 200 μl /well ABTS solution (Roche Applied Science) was added and incubated until color development. The detection at OD_{405} was performed with the Infinite M200 counter (Tecan, Salzburg, Austria). For the ELISA of the His-tagged murine DHFR, more stringent conditions were used to reduce the background signal. The blocking buffer was 1% BSA, PBS $1 \times$, pH 7.4, and the dilution of the secondary antibody, the peroxidase-conjugated goat anti-rabbit IgG, was 1:20000.

3. Results

3.1. Cloning of six different CA125-repeat units

The cloning of several repeat units from the tandem-repeat domain of CA125 was performed through the reverse-transcription and the PCR amplification of total RNA from OAW28, an ovarian cancer cell line expressing the glycoprotein [2]. Since a possible immunogenic region, the “cysteine loop”, has been previously recognized within the R-11 by O'Brien et al. [34], the encoding sequence for the R-25, expressing one of the most represented C-C region of CA125, was isolated by amplification with specific primers (Table 1, Fig. 1). Anyway, the PCR was executed in sub-stringent conditions for the annealing temperature, thereby isolating not only the encoding sequence for the R-25 but also the sequences for R-2, -7, -9, and -51. The amino acid sequences deduced from the nucleotide sequences of other isolated *E. coli* clones were redundant compared to the above-mentioned, indicating that all the possible repeats amplified with this primer pair were probably isolated. The R-11 encoding sequence was amplified with a specific primer pair and cloned to confirm literature data [34]. As expected and reported in Fig. 1, the alignment of the amino acid sequences, deduced from the nucleotide sequences from bacterial clones, shows that all the repeats present the

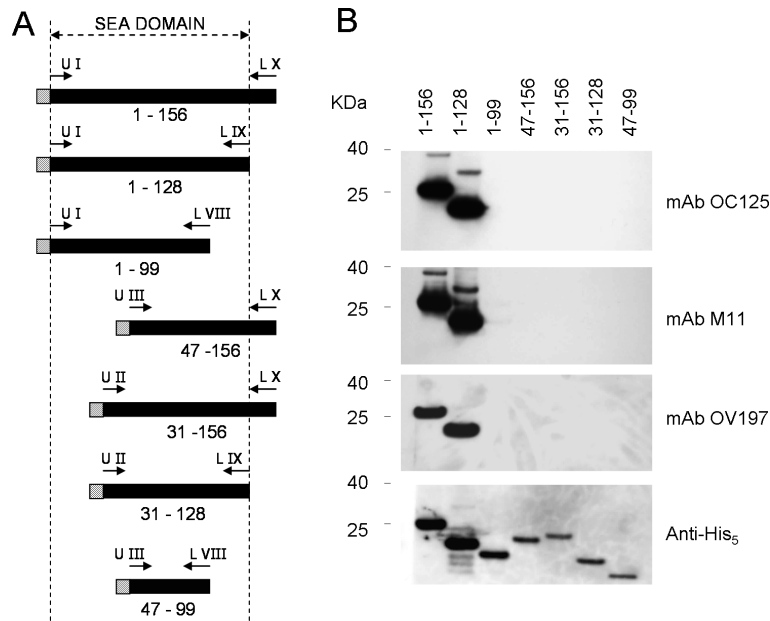


Fig. 3. Western blot analysis of recombinant regions of R-25. A: Diagram of the tested recombinant R-25 regions. Poly-histidine tags are indicated with hatched boxes. The primers used for the amplification of the encoding sequences are indicated and their sequences reported in Table 1. The numbering of the recombinant regions corresponds to the amino acid positions into the repeat. B: *E. coli* extracts containing the recombinant regions were probed with the OC125, M11, OV197 mAbs and with the anti-penta histidine as control antibody.

SEA domain [32], the “cysteine-loop” region [34], as well as amino acid substitutions among them, indicating that the corresponding conformational structures could present some differences and suggesting further investigations on the recombinant repeats.

3.2. Western blot analysis of the recombinant-repeat units

The recombinant repeats were expressed in *E. coli* clones and bacterial extracts were analyzed by Western blot analysis. The OC125, M11 and OV197 mAbs, representative of the three principal groups of CA125 antibodies [14], bound to the recombinant repeats with very different efficiencies. Unexpectedly, OC125 mAb bound very strongly to the R-25 (Fig. 2) and, as already reported in published data [34], more weakly with the R-11. Faint bands were observed for the remaining repeats. M11 mAb recognized all the repeats, with the strongest signals for R-51. Also the OV197 mAb did not recognize the recombinant CA125 repeats homogeneously: it strongly bound R-25 and R-51 and, to a lesser extent, R-2 and R-9. The OV197 mAb did not recognize R-7 and R-11.

The amino acid differences in the repeat sequences could have also determined the observed slight mobility shifts during the electrophoretic run, as discussed below.

3.3. Subcloning of R-25 deleted regions

In order to characterize the minimal region of the R-25 able to interact with CA125 antibodies, a deletion analysis was performed by subcloning partial nucleotide sequences of this repeat and by expressing the derived recombinant fragments in *E. coli*. As reported in Fig. 3A, progressive deletions were performed from both 5'- and 3'-nucleotide ends. The lengths of the overlapping regions among the fragments were sufficient to maintain putative linear epitopes [35]. The above-mentioned deleted sequences were also subcloned in frame with the murine DHFR sequence (Fig. 4A), using the pQE40 vector, to express chimeric recombinant proteins. This second subcloning was performed to better uniform the expression of the R-25 fragments, obtaining more similar protein quantities in bacterial extracts, and to analyze antibody interactions with an independent conformational arrangement.

3.4. Deletion analysis of R25 unit by Western blot

The deleted R-25s expressed by the bacterial clones were analyzed by Western blot. As shown in Fig. 3B, only the fragments containing the entire SEA domain [32] was recognized by OC125, M11 and OV197

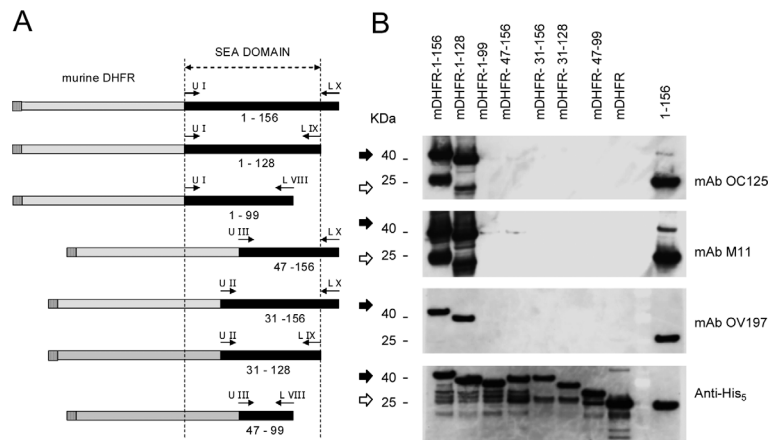


Fig. 4. Western blot analysis of chimeric regions of R-25. A: Diagram of the chimeric R-25 regions. The encoding sequences for the recombinant regions of the R-25, reported in Fig. 3, were subcloned in frame with the sequence encoding the murine DHFR. Poly-histidine tags are indicated with hatched boxes. The primers used for the amplification of the encoding sequences are indicated and their sequences are reported in Table 1. The numbering of the recombinant regions corresponds to the amino acid positions into the repeat. B: *E. coli* extracts were probed with the OC125, M11, OV197 mAbs and with the anti-penta histidine as control antibody. Lane 1–156 was loaded with the not-chimeric R-25. The black arrows indicate the chimeric constructs, while the open arrows indicate degradation products.

mAbs. Shorter fragments did not interact with antibodies at all. In spite of the same experimental conditions used for the induction of protein expression in the bacterial clones, the detection of the R-25 fragments with the anti-pentaHis antibody, that recognize the common structure of a NH-terminal poly-histidine tag, revealed that they were produced in different quantities, probably due to the different transcriptional and/or translational efficiencies of the subcloned sequences as well as to different product stabilities in the bacterial environment.

The chimeric murine-DHFR/R-25 fragments were more uniformly expressed (Fig. 4B) but, once more, only those containing the SEA domain were recognized by the CA125 antibodies. Observed bands with lower molecular weights than expected were due to bacterial proteolysis. Noteworthy, only the proteolysed fragments with the same molecular weight of the R-25 and the SEA domain were recognized by OC125 and M11 antibodies, confirming that the integrity of this domain was necessary.

3.5. R25 recognition by CA125 mAbs through ELISA

The amino-terminal poly-histidine tag of the recombinant R-25 was used to produce and purify the repeat through nickel-nitrilotriacetic acid (Ni-NTA) resin and to analyze its interactions with the anti-CA125 mAbs by ELISA using Ni-NTA coated plates. As reported in Fig. 5, also in ELISA the OC125, the M11 and the OV197 antibodies specifically bound to the repeat,

thereby confirming the Western blot data with an independent immunological method.

4. Discussion

As previously reported, the OC125 mAb, belonging to the group A of anti-CA125 antibodies, according to the ISOBM TD-1 classification [14], had shown little reactivity to the recombinant R-11 in immunoblot [34] and in antigen-capture assay [7], while, in the same experiments, the M11 mAb, belonging to the group B, reacted with high binding. Surprisingly, the OC125 mAb increased its affinity for the R-11 in the presence of competitor antibodies or after a pre-incubation with group B antibodies, indicating a conformational modulation of the structure of its epitope [7]. It has been suggested that the binding data obtained with the recombinant R-11 could be representative of CA125 interactions with antibodies and that their binding efficiency could depend on the conformational context of the repeats [7].

In this paper the subcloning and the expression in *E. coli* of several repeats, including the R-11, is reported. The R-25 was efficiently recognized not only by M11, but also by OC125, and OV197 mAbs in ELISA as well as in immunoblot assays, without any other concomitant antibody interaction. The OC125 mAb was less reactive with the R-11, as expected, and showed very low binding, if any, with the other repeats. The M11 mAb was generally reactive to all the repeats with

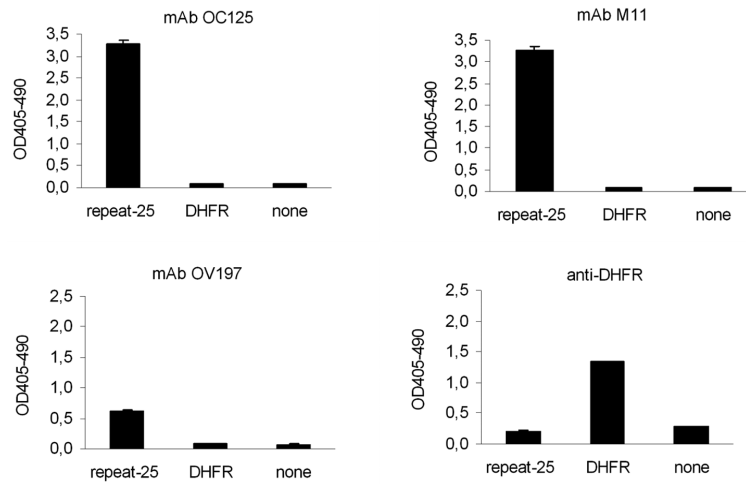


Fig. 5. ELISA analysis of R-25. *E. coli* extract containing the recombinant R-25 was tested with the OC125, M11, OV197 mAbs and with the polyclonal anti-murine DHFR as control antibody ($n = 3$). The recombinant murine DHFR was tested as mock protein.

the highest binding with R-51. The OV197 mAb bound R-25 and R-51 with high efficiency, it recognized also R-2 and R-9 and did not recognize the remaining repeats at all.

These data are in agreement with the differences observed in the amino acid sequences of CA125 repeats [34] and indicate that CA125 epitopes could be not homogeneously repeated in the tandem repeat region of the protein. This scenario is expected, considering that the repeated units are encoded by the evolving nucleotidic sequences of the tandem repeats, which may maintain or modify the epitope sequences after nucleotidic mutations. Besides, the alternative splicing of the numerous copies of the five exons encoding the repeat unit [34] may modulate the presence of epitopes. The greater number of repeats recognized by M11 mAb, respect to that recognized by OC125 and OV197 mAbs, indicates that the M11 epitope may be more represented in CA125 and could explain the different ability of these antibodies to bind the entire protein previously observed in immunoblots [14] and confirmed by our data (data not shown).

The molecular weights of the recombinant repeats observed in immunoblots were apparently not identical and often slightly different than those predicted from their amino acid sequences. The expression of the recombinant repeats in *E. coli* should rule out post-translational modifications such as glycosylation [7]. If any proteolytic cleavage occurred in the bacterial environment, the recognition of all the repeats by the anti-histidine antibody and by the M11 mAb indicates that the region of the His-tagged SEA domain, necessary also for OC125 and OV197 binding, as shown

by the deletion analysis of the R-25, has been preserved. Instead, the discrepancies among molecular weights could be due to the amino acid composition of these small fragments that could have influenced their electrophoretic run, as well as to a different negative-charge covering by SDS and/or by the poly-histidine sequence at the NH-terminal that may also retard the electrophoretic run of the recombinant repeats [21,28].

In order to map the epitopes of OC125, M11 and OV197 mAbs, a deletion analysis of R-25 amino acid sequence was performed through the subcloning and the expression in *E. coli* of partial regions of this repeat. The R-25 fragments were subcloned according to a scheme of overlapping regions from 50 up to 100 aa long, in order to maintain putative linear sequences recognized by the antibodies. The data, obtained with this deletion analysis, show that the shortest fragment recognized by anti-CA125 mAbs was an amino-terminal region 128 aa long, containing the entire SEA domain [24,31,32], indicating that the remaining 28 carboxy-terminal aa of the repeat do not participate to the formation of epitopes. This carboxy-terminal region presents a turn structure downstream the last beta sheet of the SEA domain [32], and it may be heavily O-glycosylated [34] and not prone to interaction with antibodies.

Since continuous epitopes may have a size of a maximum of 15–22 aa [35], the necessity of the entire R-25 SEA domain for the binding with OC125, M11 and OV197 mAbs, confirmed the conformational type of these epitopes and it was in agreement with the loss of M11 and OC125 epitopes after the R-11 digestion with endoprotease Lys-C or protease Asp-N [34] or with the

pattern of the *E. coli*-proteolysed fragments observed in our immunoblots. Conformational epitopes, which could not be defined by deletion mapping or overlapping synthetic peptides, may be observed in Western blots after denaturing and reducing SDS-PAGE electrophoresis, due to their possible renaturation during the transfer on membrane and their maintenance in the subsequent steps of the method, as previously reported [30,39]. The signals observed in immunoblots, derived from the entire R-25 or from the 128 aa fragment, could be thereby due to the renatured conformation of the SEA domain. The epitope conformations are very probably destroyed in the shorter deleted fragments. We can not exclude that some of the R-25 regions, encoded by the delete constructs, may interact with antibodies in a native context at least partially, and that their incorrect folding could be due to subcloning. Anyway, the data obtained with the chimeric DHFR/R-25 fragments, i.e. using a different conformational context, were superimposable to data derived from not-chimeric constructs, suggesting that the necessity of the entire R-25 SEA domain was independent from the subcloning strategy and that could be physiological.

The amino acid sequence within the R-11, known as “cysteine loop” (Fig. 1), has been reported as the site of interaction for the OC125 and M11 antibodies [34], on the basis of protease digestions of this recombinant repeat. These digestions resulted in the destruction of the epitopes without any direct evidence of antibody binding to this cysteine loop and the exact localization of the M11 and OC125 epitopes remained undetermined [7]. Nevertheless this region was synthesized as 21-mer fragment and still indicated as “peptide epitope” in further investigations [40]. Our data show that, although all the recombinant R-25 fragments encompassed the 21 aa region, they were not recognized by OC125, M11 and OV197 mAbs, neither in chimeric nor in not-chimeric constructs, with the exceptions of the fragment containing the SEA domain and the entire repeat. These data indicate that this 21 aa region is not sufficient for the interactions with the antibodies.

It is worth noting that the OV197 mAb recognized the recombinant R-25 better than the corresponding chimeric protein DHFR/R-25, and that in ELISA assays OV197 showed a lower binding-efficiency respect to OC125 and M11 antibodies. Since in chimeric constructs the NH-terminal of the R-25 is fused to the murine DHFR protein and in ELISA assays it is bound to the solid phase through the poly-histidine sequence, a steric-hindrance effect may be supposed and it might speculate that NH-terminal region of folded repeats could be in spatial proximity of OV197-epitope site.

The alignment of the amino acid sequences (Fig. 1) shows that, among the six recombinant repeats here reported, some substitutions could be responsible for their different immunological patterns; for instance, K-22, C-29, S-44 and V-93 are present only in R-25, which N-terminal region of 128 amino acids was efficiently recognized by the antibodies from all the three CA125-mAb classes. Further investigation will be focused on the mutational analysis of the recombinant repeats, in order to identify those residues that might contribute to the conformational structure of CA125 epitopes.

Taking into consideration that other amino acid sequences for the repeats are present in CA125, in addition to those reported in this work, presented data suggest an epitope map for CA125 more complex than previously supposed. A diversified presence of CA125 epitopes along the tandem-repeat domain should be considered to find more effective immuno-therapies against the epithelial ovarian cancer, since it may reflect their availability as target antigens. Besides, repeats with an immunogenic profile not much represented could promote an immune response against CA125 also in human patients, thus representing good candidates for the vaccination against ovarian cancer.

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