Introduction

Ovarian cancer (OC), with an estimated 295,000 cases and 184,000 deaths worldwide, is the 8th most prevalent cancer diagnosis and lethal gynaecological malignancy affecting a large female population (Bray et al., 2018). In Asian countries, 110,526 cases have typically been registered in the standardized OC incidence rate. Indonesia was the third-highest in OC deaths after China and India (Razi et al., 2016). Approximately 60 per cent of the women with OC already have an advanced stage of the disease at the time of presentation, and in such cases, the 5-year survival rate is just 29 per cent.

The cause for OC is still not entirely understood, but many factors that play a role in its development have been identified (Beral et al., 2008). Age, lack of birth, and family history serve as risk factors, while protective factors include parity, oral contraceptives, lactation, late menarche, and early menopause. However, direct causality is still not proven between them and OC (Tsilidis et al., 2011). Various factors, including atypical clinical symptoms, insufficient early detection modalities, late-onset diagnosis, chemoresistance, recurrence, and metastatic spread, lead to high mortality of ovarian cancer (Burges and Schmalfeldt, 2011; Chen et al., 2017; Klapdor et al., 2017).

Approximately ninety per cent of OC are of epithelial origin. Based on the histological and molecular findings, OC was classified into type 1 and type 2. Type 1 is slow-growing, low grade, and genetically stable, while type 2 is more aggressive and variably alters the p53 molecular levels (Malpica and Wong, 2016). According to the current WHO classification, serous tumours are the most common histologic type of female genital tract tumours (Kurman et al., 2014). Low-grade serous carcinoma (LGSC) grows indolently from precursor lesions: serous cystadenoma (SC) to a borderline serous tumour (BST) (Hatano et al., 2019). The micropapillary pattern with or without invasion is a histopathological hallmark used to differentiate between BST and LGSC (Kurman et al., 2014). In comparison, high-grade serous carcinoma (HGSC) has been demonstrated to derive from a stepwise progression of intraepithelial tumour serous tubal lesions (STIC) (Lee et al., 2007). KRAS-BRAF and TP53 mutations are involved in these two entities, respectively (Tsuchida et al., 2016; Hatano et al., 2019). Their characterization is based on a histologic analysis showing that tumour cells resemble a sort of epithelial-like cells that most likely come from the fallopian tube epithelial (FTE) or ovarian surface epithelial (OSE)

Objective: To analyze the role of cancer stem cells (CSC) in ovarian carcinogenesis through the identification of CD133 expression in the normal ovary (NO), serous cystadenoma (SC), borderline serous tumour (BST), low-grade serous carcinoma (LGSC), and high-grade serous carcinoma (HGSC). Materials and methods: A total of 48 tissue samples contain 5 NO, 10 SC, 5 BST, 8 LGSC, and 20 HGSC were stained with anti-CD133 antibody by immunohistochemical protocol. The difference in the H-score of CD133 expression between groups and their relationship to age, histomorphology, and localization was analyzed. Results: CD133 expression varied among tumor groups, with clinicopathologic parameters showing diverse associations (age p = 0.773; histomorphology p = 0.001; and localization p = 0.026). The comparison of CD133 H-scores differed significantly between each group (p = 0.0031), in which precursor and malignant lesions possessed more robust CD133 expression. Conclusion: The presence of CD133 cellular expression and localization in different types of serous ovarian tumours suggests that these markers are involved in ovarian tumorigenesis.

Keywords: CD133- cancer stem cells- carcinogenesis- ovarian cancer
(Kurman et al., 2014). Both cells express cancer stem cells (CSC) markers, including ALDH1, LGR5, LFE1, CK6B, and CD133 (Auersperg, 2013). CD133 or Prominin-1 is a membrane-anchored cell surface protein first discovered in mouse and human stem cells (Shmelkov et al., 2005), which indicates the presence of cancer stem cells in tumorous tissue (Barzegar et al., 2019). It is often challenging to predict the likelihood of malignant transformation based on histopathological findings alone. Therefore, it is essential to include an additional measure to assess potential malignant transformation in an ovarian precursor lesion. The identification of CD133+ cells provides insight into tumour biology related to metabolism, apoptosis, autophagy, tumorigenesis, metastases, and chemoresistance (Li, 2013). It is also expected to have practical implications for assessing the risk of malignancy in precursor lesions. Thus, this study aimed to analyze the role of CSC in ovarian carcinogenesis through CD133 expression in normal ovaries, precursor lesions, and malignant lesions.

Materials and Methods

The research design was an observational study with a cross-sectional approach through histomorphological and immunohistochemical analysis of precursors and malignant lesions. We analyzed 48 samples consisted of 5 normal ovaries, ten SC, five BST, eight LGSC, and 20 HGSC. All samples were embedded paraffin and obtained from patients in three anatomical pathology centres in Makassar (Wahidin Sudirohusodo Hospital, Hasanuddin University Hospital, and Makassar Pathology Diagnostic Center). Samples were collected from January 2017 to December 2020. The use of stored biological material has been approved by The Research Ethics Committee of the Faculty of Medicine Universitas Hasanuddin.

Histomorphological analysis

Specimens were first fixed in 10% buffered formalin, then embedded in paraffin, sectioned at 5 µm, stained with hematoxylin-eosin, and placed on glass slides for histopathological study. Samples have been classified according to the WHO Classification of Female Genital Tumours 5th edition.

Immunohistochemical procedure

Tissue samples at 3 µm were sectioned from paraffin-embedded material. The entire slides were treated according to standard protocols, and serial cuts were used for immunohistochemical reactions (IHC). After deparaffinization in xylene and rehydration in various ethanol concentrations, slides were placed in 3% hydrogen peroxide for 3 minutes then washed in tap water. Heat-induced epitope retrieval was then performed by putting samples into the microwave for 10 minutes. Specimens were then allowed at room temperature and washed with Tris Buffer Saline and 1% protein blocking solution for 10 minutes. All specimens were then incubated in primary antibody (1:200) for 90 minutes at room temperature. The samples were then incubated in secondary antibody (Ultratek Complete HRP Anti-Polyvalent – LSAB) for ten minutes. Next, all samples were then incubated with chromogen Diaminobenzidine (DAB) and washed in running water for 5 minutes, then immersed in a solution of Hematoxylin Modified Lillie’s Mayer as counterstaining. Finally, all slides were then covered on a deck glass and further analyzed under the Microscope Binocular Olympus CX-43. Each reaction was accompanied by positive control (pancreas). Negative control (absence of primary antibody) was used in conjunction with the incubation of the samples.

Quantitative Analysis

Two gynaecological pathologists analyzed CD133 expression without knowing the clinical data of each other. Assessment based on the percentage of stained cells (extensity) in 10 ‘hotspot’ high power field (0-100), intensity (0-3), and histochecmic score (H-Score) (0-300) by multiplying extensity and intensity. CD133 expression is categorized as a low or high expression based on the median H-score determined as a cut-off value (Keymoosi et al., 2014). Positive expression of CD133 will appear brown stained on the membrane and cytoplasm of tumour cells. The percentage of stained cells was divided into 3 levels: 1 (0% CD133 + cells), 2 (<10% CD133 + cells), and 3 (>10% CD133 + cells) (Cioffi et al., 2015). The CD133 staining intensity levels were classified into four categories: 0 (no stained/negative cells), +1 (weak staining or only visible with 40x objective lens magnification), +2 (moderate or visible staining with 10x objective lens magnification), +3 (strong or visible stain with 10x objective lens magnification) (Mardani et al., 2020). All slides were observed at x400 magnification using a light microscope (Olympus CX-43).

Statistical Analysis

All data were analyzed using GraphPad Prism software version 6. Values are expressed in mean ± SD. The Kruskal-Wallis test, followed by the Mann-Whitney test, was used to assess the mean H-score difference between the test groups. A p-value <0.05 was considered statistically significant.

Results

Sample Characteristics

Samples were obtained from 48 cases consisting of 5 normal ovaries, 10 SC, 5 BST, 8 LGSC, and 20 HGSC. The mean age in the SC and BST premalignant lesion groups were 42.2 ± 8.63 SD and 41.6 ± 15.8 SD, ranging between 24-56 years and 26-66 years, respectively. Meanwhile, the age range of LGSC and HGSC malignant lesions was between 23-58 years and 24-77 years with mean age 42 + 13.4 SD and 50.4 + 12.9 SD. As shown in table 1, there was no significant age difference between the CD133-low expression tumour group and the CD133-high expression group.
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p-value=0.026. The data indicates a relationship between histomorphology and localization.

Immunohistochemical profiles of CD133 in ovarian tumours

The data showed significant differences in the site of CD133 expression based on the type of histomorphology with a p-value=0.026. The data indicates a relationship between histomorphology and localization.

Figure 1. Representative Samples of CD133+ Cells among Tumour Group. (A) Positive control in human pancreas tissue; (B) Normal ovary showed positive expression located in stromal cells with apical/membranous pattern; (C) Serous Cystadenoma expressed CD133 in epithelial lining cells with membranous pattern; (D) Borderline Serous Tumor showed positive CD133 expression diffuse strong cytoplasmic pattern; (E) Low-grade Serous Carcinoma; and (F) High-grade serous carcinoma expressed CD133+ve in tumour cells, respectively. Note that staining intensity and extensity in HGSC are greater than LGSC. Magnification 400x; Scale bar 100 µm.

Figure 2. CD133 H-score Comparison Regarding Histomorphology. There was a statistically significant difference among the groups of tumours (Kruskal-Wallis test p=0.0031). *Post-Hoc analysis Mann-Whitney showed a significant difference between SC vs. BST p=0.0366, BST vs. LGSC p=0.0451, and LGSC vs. HGSC p=0.0221.
We identified CD133+ cells in normal ovaries, benign and precursor lesions, and malignant lesions (Figure 1A-1F). It appeared that the intensity and the percentage of the area of CD133 stained varied in each group. The most robust expression was observed in BST, HSGC, and LGSC, located in the cytoplasm. The SC lesion shows weak CD133 expression on the membrane or apical tumour cells. The expression of CD133 in normal ovaries showed a membrane staining pattern in the stromal area with weak intensity. Furthermore, we analyzed the CD133 positive rate values in each histomorphological group (Table 1). Positive rates> 80% were indicated by the SC and BST precursor lesion groups and the LGSC and HGSC malignant lesion groups, where BST and HGSC showed the highest values.

We compared H-scores for CD133 expression between the normal ovaries, benign, precursor, and malignant lesions. The results showed that there was a statistically significant difference between each tumour group p 0.0031. Subsequent Mann-Whitney analysis revealed significant H-score differences between the three groups, namely SC vs. BST p 0.0366, BST vs. LGSC p 0.0451, and LGSC vs. HGSC p 0.0221, while NO vs. SC not statistically significant p 0.1522 (Figure 2).

| Characteristics of Sample | n (%) | CD133 Expression | p |
|---------------------------|-------|------------------|---|
| Age (years)               |       | Low n (%)        | High n (%)    |   |
| Mean±SD                   | 47.23 (12.9) | 47.71 (13.6) | 46.75 (12.5) | 0.802* |
| <47                       | 25 (52.1)   | 13 (54.2)       | 12 (50)      | 0.773b |
| >47                       | 23 (47.9)   | 11 (45.8)       | 12 (50)      |   |
| Histo-Morphology          |       |                 |               |   |
| Normal ovary              | 5 (10.4)   | 5 (20.8)        | 0 (0)        | 0.001c |
| Serous cystadenoma        | 10 (20.8)  | 7 (29.2)        | 3 (12.5)     |   |
| Borderline serous cystadenoma | 5 (10.4)   | 0 (0)          | 5 (20.8)     |   |
| Low grade serous carcinoma | 8 (16.7)    | 6 (25)         | 2 (8.3)      |   |
| High grade serous carcinoma | 20 (41.7) | 6 (25)         | 14 (58.3)    |   |
| Localization              |       |                 |               | 0.026d |
| Apical or membranous      | 13 (27.1)  | 8 (33.3)        | 5 (20.8)     |   |
| Cytoplasmic               | 29 (60.4)  | 10 (41.7)       | 19 (79.2)    |   |
| NA*                       | 6 (12.5)   | 24 (50)         | 24 (50)      |   |

*NA, Not Assessable; * Unpaired T-Test; * Chi-square; * Fisher’s Exact Test

We identified CD133+ cells in normal ovaries, benign and precursor lesions, and malignant lesions (Figure 1A-1F). It appeared that the intensity and the percentage of the area of CD133 stained varied in each group. The most robust expression was observed in BST, HSGC, and LGSC, located in the cytoplasm. The SC lesion shows weak CD133 expression on the membrane or apical tumour cells. The expression of CD133 in normal ovaries showed a membrane staining pattern in the stromal area with weak intensity. Furthermore, we analyzed the CD133 positive rate values in each histomorphological group (Table 1). Positive rates> 80% were indicated by the SC and BST precursor lesion groups and the LGSC and HGSC malignant lesion groups, where BST and HGSC showed the highest values.

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Table 2. The Distribution of CD133 Positive Rates among Tumour Groups

| Group of Tumours          | N  | CD133 Expression |  Positive Rates (%) |
|---------------------------|----|------------------|---------------------|
|                           |    | Negative | Positive |               |
| Normal Ovary              | 5  | 2        | 3        | 60               |
| Serous Cystadenoma        | 10 | 2        | 8        | 80               |
| Borderline Serous Tumour  | 5  | 0        | 5        | 100              |
| Low-Grade Serous Carcinoma| 8  | 1        | 7        | 87.5             |
| High-Grade Serous Carcinoma| 20 | 1        | 19       | 95               |
| Total                     | 48 | 6        | 42       | 87.5             |

Discussion

This study presented a relatively younger mean age in the premalignant lesion group than the malignant lesion group. (Longacre et al., 2005) noted that the mean age of patients with serous tumours of low malignant potential was 42.3 years. In malignant, Arora et al., (2018) reported age at diagnosis to be 50-79 years, whereas our findings showed the mean age in the HGSC group falls within this range. These indicate the age trend of patients with precursor lesions and malignant ovarian epithelial lesions in the 4th to 5th decade of life. Although the relationship between age and outcome of ovarian cancer is unclear, several studies have reported that younger age showed better outcomes than older age due to aggressive treatment (Ries, 1993; Arora et al., 2018). Other risk factors that also play a role include menstrual aspects, endometriosis, family history, and BRCA mutations, while parity, contraceptive methods, and lactation are protective factors (Momenimovahed et al., 2019).

Our evaluation of the staining pattern with CD133 expression in the various ovarian tumour groups showed a statistically significant difference (see Table 1). The apical or membranous staining pattern was predominantly found in normal ovarian located in the stromal spindle cells and SC tumour cells. In contrast, malignant lesions, especially HGSC, showed diffuse staining patterns in the cytoplasm. In line with these findings, (Huang et al., 2015) reported CD133 expression in the cytoplasm and nucleus of tumour cells associated with a poor prognosis in non-small cell lung cancer. Ferrandina et al., (2009) also noted a tendency for ovarian cancer with a CD133 diffuse cytoplasmic staining pattern to have a worse prognosis than with the apical cytoplasmic pattern. CD133 is a transmembrane protein in non-tumorous cells, while in tumorous lesions, it can be internalized into the

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CD133 cellular expression and localization in various groups of ovarian tumours of serous epithelial origin, ranging from benign, borderline, and malignant entities, indicates the involvement of these markers in ovarian cancer tumorigenesis. However, this study has limitations because it only uses a single CSC marker. Histopathologic determination of tumour grading still has an aspect of subjectivity. The CD133 immunohistochemical modality, together with histopathological findings on premalignant precursor lesions, can better identify and assess malignancy risk.

### Author Contribution Statement

DI, RM, BJN contributed to the conceptualization and design of the method; DI, RM, BJN, AAZ engaged with the curation of data, analysis, and interpretation of the findings; MG and SW reviewed it extensively for intellectual content and editing; All authors reviewed and approved the final version of the article.

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DI, RM, BJN contributed to the conceptualization and design of the method; DI, RM, BJN, AAZ engaged with the curation of data, analysis, and interpretation of the findings; MG and SW reviewed it extensively for intellectual content and editing; All authors reviewed and approved the final version of the article.
University Hospital.

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**Study Approval**

This manuscript is a part of an approved DI’s thesis on the academic program for anatomic pathology specialist.

**Availability of Data**

The datasets used in this study are available upon reasonable request from the corresponding author.

**Ethical approval**

The Ethics Committee of the Faculty of Medicine, University of Hasanuddin, Makassar, Indonesia permitted this study (Registry No. 309/UN4.6.4.5.31/PP36/2021) with a waiver of informed consent.

**Conflict of Interest**

All authors state that they have no conflicting interests.

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