Frequent gain of the human telomerase gene TERC at 3q26 in cervical adenocarcinomas

S Andersson1, K-L Wallin2, A-C Hellström3, LE Morrison4, A Hjerpe5, G Auer5, T Ried6, C Larsson*2 and K Heselmeyer-Haddad6

1Department for Clinical Science, Intervention and Technology, Division of Obstetrics and Gynecology, Karolinska University Hospital-Huddinge, Karolinska Institutet, Stockholm SE-141 86, Sweden; 2Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital-Solna, CAM L8.0, Stockholm SE-171 76, Sweden; 3Department of Gynecologic Oncology, Radiumhemmet, Karolinska University Hospital-Solna, Stockholm SE-171 76, Sweden; 4Abbott Molecular, 1300 East Touhy Avenue, Des Plaines, IL 60018, USA; 5Department of Oncology-Pathology, Karolinska University Hospital Solna, CKK, Stockholm SE-171 76, Sweden; 6Genetics Branch, Center for Cancer Research, National Cancer Institute/NIH, Building 50, Room 1408, 50 South Drive, Bethesda, MD 20892-8010, USA

The level of genomic amplification of the human telomerase gene TERC, which maps to chromosome band 3q26, was determined in primary cervical adenocarcinomas. Interphase nuclei prepared from archival material of 12 primary cervical adenocarcinomas, eight of which were human papillomavirus positive, were hybridised with a triple colour probe set specific for centromeres of chromosomes 3 and 7 and the TERC gene. We observed high proportions of nuclei with increased absolute copy numbers for TERC in all tumours (mean 3.3; range 2.3–5.2). Amplification of the human telomerase gene TERC is a consistent aberration in cervical adenocarcinomas. Therefore, application of our probe set may provide an objective genetic test for the assessment of glandular cells in Pap smears and hence for the diagnosis of cervical adenocarcinomas.

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Cervical cancer is a disease that mainly affects women in the forth or fifth decade of life. According to the International Agency for Research on Cancer (IARC), it is the second most common cancer in women worldwide, and in developing nations it is the most common cancer in women (Parkin et al, 2001). Two main types of cervical cancer are described, that is, squamous cell carcinoma and adenocarcinoma. Cervical adenocarcinomas comprise 20% of all cervical cancers that are diagnosed (Vizcaino et al, 1998). Importantly, in the last few decades the incidence of cervical adenocarcinoma among young women has been steadily increasing (Vizcaino et al, 1998; Bergström et al, 1999; Smith et al, 2000; Sasiene and Adams, 2001); however, the reasons for this increase are presently unknown. Applications of clinical screening programmes that have led to a decrease in squamous cell carcinoma have only shown a limited effect on the occurrence of adenocarcinomas. This is owing to the fact that the sensitivity for detecting precursor lesions of adenocarcinomas is much lower than for the detection of precursor cells of squamous carcinomas. This can be partly explained by the difficulty in the morphological assessment of highly differentiated glandular cells, which can be derived from adenocarcinomas of the endocervix. In addition to these difficulties, the endocervix is less accessible for cytological sampling (Bergström et al, 1999).

Epidemiological and molecular biological studies have shown that infection with high-risk human papillomavirus (HPV) is an important aetiological agent in the pathogenesis of cervical adenocarcinoma (Bosch et al, 1995; Bosch and Munoz, 2002). More than 99% of squamous cell carcinomas harbour oncogenic types of HPV, with HPV 16 being the most frequently encountered (Walboomers et al, 1999). Partly in contrast, we and others have found HPV DNA in 30–90% of cervical adenocarcinomas, with HPV 18 as the predominant type (Tenti et al, 1996; Andersson et al, 2003a, b; Clifford et al, 2003; Zielinski et al, 2004; Castellsague et al, 2006).

Although tightly linked to cervical cancer development, HPV infection alone is not sufficient for oncogenic transformation, and other factors than HPV are required for the malignant transformation of cervical adenocarcinomas (Skyldberg et al, 1999; Pirog et al, 2000). The application of cytogenetic and molecular cytogenetic techniques to study genomic alterations have identified frequent gains involving the long arm of chromosome 3 (Yang et al, 2001). This observation is in concordance with previous findings of recurrent 3q amplification in squamous cell carcinomas (Heselmeyer et al, 1996, 1997a). The value of detection of genomic amplification of 3q as a biomarker for progression during uterine tumorigenesis was therefore evaluated in several studies. The results showed that amplification of TERC is almost invariably found in Pap smears assessed as HSIL (i.e., high squamous intraepithelial lesions). In addition, a more recent study un-
ambiguously established the central role of 3q for progression from low-grade dysplastic lesions to higher grades and to invasive carcinomas and showed that the gain of 3q can occur in morphologically normal Pap smears of women who developed cervical carcinomas after only a short latency (Heselmeyer-Haddad et al, 2003, 2005). Gain of chromosomal region 3q is also recurrently observed in other types of cancer, including tonsillar cancer and carcinomas of the anogenital region that are both associated with HPV DNA (Heselmeyer et al, 1997b; Dahlgren et al, 2003; Stoltzfus et al, 2005).

In this study, we have determined TERC copy number gain in cervical adenocarcinomas using interphase fluorescence in situ hybridisation (FISH) on cytopsins prepared from single-cell suspensions of disintegrated archival paraffin-embedded tissue and correlated the findings to the presence of HPV infection and the clinical course.

PATIENTS AND METHODS

Tumour material

The study includes formalin-fixed paraffin-embedded tumour tissue specimens from 12 primary cervical adenocarcinomas diagnosed and surgically treated at the Karolinska University Hospital-Huddinge during 1992 – 2000 (Table 1). All tumour cases were identified from the Swedish Central Cancer Registry organised by the National Board of Health and Welfare. This registry includes all cases of malignant tumours diagnosed histopathologically after 1959, whereby each tumour is identified by a topographical and histopathological code. All tumour samples were collected with informed consent and approval from the local ethics committee.

The clinical information for each case is detailed in Table 1 and has been published previously (Andersson et al, 2003a). The histopathological diagnosis was based on the WHO criteria. The 12 tumours were classified as cervical adenocarcinomas and all were without any squamous cell component. Furthermore, the four HPV-negative tumours (T4, T5, T8, T9) were histopathologically re-evaluated and revealed typical morphology of cervical adenocarcinoma in agreement with the initial diagnosis. Immunostainings with vimentin and carcinoembryonic antigen (CEA) were carried out. Cases T4, T5 and T8 were strongly positive for CEA and negative for vimentin, in agreement with the common pattern for adenocarcinoma of cervical origin as reported by McCluggage et al (2002). Case T9 showed atypical staining pattern (CEA was negative and vimentin positive); however, the morphology was strongly suggestive of cervical origin as judged by several independent histopathologists. Seven tumours were well differentiated (58%), one was moderately differentiated (8%) and four tumours were poorly differentiated (33%). Clinical staging was according to the International Federation of Gynecology and Obstetrics (FIGO) classification for cervical cancer (Benedet et al, 2000). At initial diagnosis, 10 tumours were classified as stage I (83%) and two tumours as stage II (17%). Two patients initially exhibited lymph node involvement (17%). All patients were previously sampled for Pap smear analysis. All but one Pap smear (which was evaluated as showing signs of cellular inflammation) were assessed as normal at the time of the most recent cytological evaluation (Table 1). Re-evaluation of the Pap smears confirmed the initial diagnoses in all cases (Andersson et al, 2003a). All patients were retrospectively followed up from the time of diagnosis until June 2005, and disease recurrence and survival data recorded.

HPV status

Results from HPV screening analyses have been previously published for all cases (Andersson et al, 2003a). Briefly, the analyses were performed on extracted DNA obtained from sections of paraffin blocks, of which the preceding section had been used for morphological diagnosis. A fragment of 139–148 bp was amplified from the L1 region with GP5+/GP6+ primers, HPV typed by direct DNA sequencing and comparison to known HPV sequence databases using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST). Eight of the 12 tumours were thus found to be HPV-positive; five tumours were infected with HPV 18, two with HPV 16 and one with HPV 45 (Table 1). In the remaining four tumours, HPV was not detected.

Preparation of nuclei suspensions for FISH analysis

Single-layer nuclei preparations for interphase FISH hybridisations were prepared using the Hedley method with modifications (Castro et al, 1993). A 50-μm section was cut from each of the 12 formalin-fixed paraffin-embedded tumour tissue samples. After deparaffinisation in xylene, the section was rehydrated in an ethanol series and in distilled water, and the section was disintegrated in 500 μl of 0.1% protease/1× PBS (Protease : Type XXIV, Bacterial, P 8038, Sigma, St Louis, MO, USA and Dulbecco’s 1× PBS, Life Technologies, Rockville, MD, USA) at 45°C for 45–60 min. The reaction was stopped by adding 500 μl 1× PBS at room temperature. The sample was then filtered through a nylon membrane (CN 051, DAKO, Glostrup, Denmark), centrifuged and

Table 1 Clinical details and HPV analysis of the 12 adenocarcinomas in the study

| Case no. | Cancer diagnosis | Last Pap smear year/ result | Age at diagnosis (years) | Tumour stage | Tumour differentiation | Lymph node involvement | HPV infection | Time (years) | Outcome |
|----------|------------------|-----------------------------|--------------------------|--------------|-----------------------|------------------------|---------------|--------------|---------|
| 1        | 1992             | 1990/normal                 | 30                       | 1B           | Poor                  | No                     | HPV 16        | 13           | AwoD    |
| 2        | 1992             | 1989/normal                 | 46                       | 1B           | Poor                  | No                     | HPV 18        | 13           | AwoD    |
| 3        | 1995             | 1992/normal                 | 37                       | 1B           | Well                  | No                     | HPV 18        | 10           | AwoD    |
| 4        | 1997             | 1987/normal                 | 39                       | 1B           | Well                  | Yes                    | Not detected  | 8            | AwoD    |
| 5        | 1992             | 1978/normal                 | 50                       | 2B           | Poor                  | Yes                    | No            | 13           | AwoD    |
| 6        | 1994             | 1992/normal                 | 32                       | 1B           | Poor                  | No                     | HPV 18        | 11           | AwoD    |
| 7        | 2000             | 1997/normal                 | 62                       | 1B           | Well                  | No                     | HPV 45        | 4            | DoD     |
| 8        | 2000             | 1998/normal                 | 64                       | 1B           | Poor                  | No                     | Not detected  | 5            | AwoD    |
| 9        | 1999             | 1996/normal                 | 63                       | 2B           | Moderate              | Yes                    | Not detected  | 6            | AwoD    |
| 10       | 2000             | 1999/normal                 | 53                       | 1A           | Well                  | No                     | HPV 16        | 5            | AwoD    |
| 11       | 1997             | 1996/normal                 | 45                       | 1B           | Well                  | No                     | HPV 18        | 7            | DoD     |
| 12       | 1993             | 1992/normal                 | 49                       | 1A           | Well                  | No                     | HPV 18        | 11           | AwoD    |

AwoD = alive without disease; DoD = dead of disease; HPV = human papillomavirus.
resuspended in 1 × PBS. Cytospin slides were prepared using a Shandon Cytospin® centrifuge and fixed in an ethanol series.

**FISH**

Triple-colour FISH analysis was performed on each case using the following probe set: a centromere-specific probe for chromosome 7 (CEP7™); a centromere-specific probe for chromosome 3 (CEP3) and a contig consisting of four overlapping BAC clones containing the TERC gene at chromosomal location 3q26. All probes were obtained from Vysis/Abbott Molecular Inc. (Des Plaines, IL, USA). The details for this probe set, its sensitivity and specificity, as well as experimential conditions were published previously (Heselmeyer-Haddad et al., 2003). In short, CEP7 was labelled with Spectrum Aqua™ (SA), CEP3 with Spectrum Green™ (SG) and the TERC contig with Spectrum Orange™ (SO), using chemical labelling as described (Bittner et al., 1996). Before hybridisation, the cytospin slides were pretreated with a pepsin digestion and fixed in an ethanol series. Slides were denatured in 70% formamid/2 × SSC for 3.5 min at 80°C. The probes were denatured according to the manufacturer’s recommendations. After overnight hybridisation at 37°C, the slides were first washed four times in 50% formamid/2 × SSC at 45°C (once for 3 min and three times for 7 min), followed by washes in 2 × SSC at 45°C for 5 min and in 2 × SSC/0.1% NP40 at 45°C for 5 min. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI), and subsequently embedded in an antifade solution.

**Scoring of FISH results**

Fluorescence *in situ* hybridisation and image analyses were carried out using a Leica DM-RXA fluorescence microscope (Leica, Wetlitz, Germany) equipped with custom optical filters for DAPI, SA, SG and SO (Chroma Technologies, Brattleboro, VT, USA) and ×40 Plan Apo (NA 1.25) objective. Images were taken in areas of optimal cell density with minimal cellular clumps using an ORCA ER (1307 E1FX) digital camera (Hamamatsu, Bridgewater, NJ, USA). Leica Q-Fluoro was used to acquire multifocus images for each of the DAPI, SA, SG and SO optical filters. Ten to 16 images were acquired and signal enumeration was performed on these digital images for 208–641 nuclei for each case. The counted signals were listed and evaluated in Excel-based customised software.

Nuclei that could not be evaluated (e.g., because of insufficient hybridisation or overlapping nuclei) were excluded from further analysis. The results for all ‘countable’ nuclei were registered in relocation charts in the form of patterns for the entire probe panel. For example, the pattern (2-3-3) refers to two signals for CEP7, three signals for CEP3 and three signals for TERC in a given nucleus. Nuclei with normal signal numbers for the three probes (i.e., 2-2-2) were recorded as ‘diploid’, and nuclei with four signals for each probe (pattern 4-4-4) were considered ‘tetraploid’. The background level for the CEP7–CEP3–TERC probe panel was previously evaluated on cytological slides that contained nuclei from normal cervical cells (Heselmeyer-Haddad et al., 2003). This confirmed that deviation from the expected (2-2-2) pattern was seen in less than 2% of normal cells.

**RESULTS**

**Frequent gain of TERC in cervical adenocarcinomas**

Twelve cervical adenocarcinomas (Table 1) were evaluated for copy number changes of the TERC locus at chromosomal band 3q26 using interphase FISH. The three-colour probe panel consisting of CEP7–CEP3–TERC was simultaneously hybridised to cytospin slides with interphase nuclei prepared from formalin-fixed tissue sections. All cases were successfully hybridised and analysed, whereby 208–641 nuclei per case were scored. Figure 1 shows representative hybridisations. Both normal ‘diploid’ patterns (2-2-2) and nuclei with nondiploid patterns were observed. The results are summarised in Table 2. In all 12 tumours, a significant proportion of ‘nondiploid’ nuclei was detected, and none of the tumours exhibited >50% ‘nondiploid’ nuclei. In the individual tumours, nuclei with >2 TERC signals were found in similar frequencies as cells with ‘nondiploid’ pattern, and almost all nuclei with ‘nondiploid’ pattern exhibited >2 TERC signals. In the ‘nondiploid’ nuclei, a ‘tetraploid’ pattern (4-4-4) was not commonly observed. In 10 of the tumours, less than 1% of the nuclei exhibited (4-4-4) and in two cases (4-4-4) was seen in 4 and 12%, respectively (Table 2; exemplified for case 3 in Figure 2). Thus, gain of TERC was present in all tumours studied and was not an effect of ‘tetraploid’ status.

**Intratumour heterogeneity for gain and amplification of the TERC locus**

We observed different FISH patterns in the individual cases. For several of the tumours, one or a few predominating patterns were seen (exemplified for cases 1, 3 and 12 in Figure 2). In addition, all cases exhibited varying proportions of different but recurrent ‘nondiploid’ patterns. Comparison of signal numbers recorded for TERC vs CEP7 and CEP3 showed increased relative TERC copies in the 12 cases studied (range 1.04–2.0; Table 2). The absolute mean number of TERC signals per nuclei ranged between 2.3 (case 11) and 5.2 (case 10), and in five of the tumours nuclei with more than 10 signals were repeatedly encountered (Table 2). Figure 1 illustrates nuclei of case 10 with amplifications of up to 20 TERC signals and more, together with 5–6 signals for CEP3 and CEP7 (Figure 1G and H), which shows that TERC can be highly amplified in certain cases. However, the more common pattern observed in most cases are low-level copy numbers of TERC, often on a still diploid background with patterns that can be reconciled with extra chromosomes of 3 or an isochromosome 3q formation.

**Gain of TERC and HPV infection**

The clinical and follow-up information as well as HPV status of the 12 cervical adenocarcinomas are provided in Table 1. Eight cases were HPV-positive and in the other four HPV was negative. Very high proportions of ‘nondiploid’ nuclei with >2 TERC signals were demonstrated in HPV-positive (83–99%) as well as HPV-negative (86–99%) tumours. The absolute numbers of TERC signals per nucleus were similarly increased in HPV-positive (mean 3.4; range 2–22) and HPV-negative (mean 3.3; range 2–12) tumours. No apparent associations between TERC copy numbers and clinical characteristics, such as age at diagnosis, stage, differentiation, lymph node involvement or outcome at follow-up, were noted. Thus, gain of TERC is characteristic of cervical adenocarcinomas per se.

**DISCUSSION**

In our study, we analysed a series of 12 primary cervical adenocarcinomas for gain or amplification of the human telomerase gene TERC, which maps to chromosome band 3q26. Eight of the 12 carcinomas (67%) were HPV-positive (five tumours were positive for HPV 18, two for HPV 16 and one for HPV 45) (Skyldberg et al., 1999; Andersson et al., 2003a). We then used FISH with a custom-designed probe panel that includes the human telomerase gene TERC, which maps to chromosome band 3q26. Eight of the 12 carcinomas (67%) were HPV-positive (five tumours were positive for HPV 18, two for HPV 16 and one for HPV 45) (Skyldberg et al., 1999; Andersson et al., 2003a). We then used FISH with a custom-designed probe panel that includes the human telomerase gene TERC to assess the potential of this genetic marker to ameliorate the morphological diagnosis of cervical adenocarcinomas. This is the first study that reports the consistent gain of the human telomerase gene TERC in cervical adenocarcinomas.
Yang et al. (2001) analysed 20 cases of cervical adenocarcinomas with CGH and found that genetic alterations were evident in most of the cervical adenocarcinomas, including the gain of 3q, which was detected in 70% of these 20 tumours. From the findings of similar 3q amplifications in adenocarcinomas as reported for squamous cell carcinoma, it was proposed that similar

Figure 1  Interphase FISH analysis showing gain of TERC in 3q26 in cervical adenocarcinomas. (A) Chromosomal location of the triple-colour probe set: CEP 7 (labelled with Spectrum Aqua, which is pseudocoloured in yellowish-white in panels B, C, E and G), CEP 3 (Spectrum Green, pseudocoloured in green in panels B, C, E and G), and TERC (Spectrum Orange, pseudocoloured in red). (B) Normal nuclei with the expected pattern of two signals per probe per nucleus (2-2-2). (C and D) HPV-negative case number 4, (E and F) HPV-infected case number 6 and (G and H) HPV-positive case number 10. All cases exhibit varying extents of gain of TERC. The cell clusters in C, E and G and in D, F and H show identical cells with either the triple-colour probe set shown or with the TERC probe only, respectively.
| Case no. | Nuclei counted | 'Diploid' (2-2-2) (%) | 'Nondiploid' > 2 TERC (%) | 'Tetraploid' (4-4-4) (%) | TERC | CEP3 | CEP7 | Relative TERC vs |
|---------|----------------|-----------------------|---------------------------|------------------------|-------|-------|-------|-----------------|
| 1       | 376            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 91              |
| 2       | 391            | 31                     | 58                       | 67                     | 97    | 92    | 94    | 93              |
| 3       | 215            | 27                     | 40                       | 33                     | 97    | 91    | 94    | 91              |
| 4       | 466            | 40                     | 68                       | 56                     | 97    | 92    | 94    | 95              |
| 5       | 391            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 97              |
| 6       | 215            | 27                     | 40                       | 33                     | 97    | 91    | 93    | 98              |
| 7       | 466            | 40                     | 68                       | 56                     | 97    | 92    | 94    | 91              |
| 8       | 391            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 92              |
| 9       | 215            | 27                     | 40                       | 33                     | 97    | 91    | 93    | 92              |
| 10      | 466            | 40                     | 68                       | 56                     | 97    | 92    | 94    | 95              |
| 11      | 391            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 97              |
| 12      | 215            | 27                     | 40                       | 33                     | 97    | 91    | 93    | 91              |

**Table 2** Results from interphase FISH analysis of the 12 cervical adenocarcinomas

**Signs per cell: mean (range)**

| Case no. | Nuclei counted | 'Diploid' (2-2-2) (%) | 'Nondiploid' > 2 TERC (%) | 'Tetraploid' (4-4-4) (%) | TERC | CEP3 | CEP7 | Relative TERC vs |
|---------|----------------|-----------------------|---------------------------|------------------------|-------|-------|-------|-----------------|
| 1       | 376            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 91              |
| 2       | 391            | 31                     | 58                       | 67                     | 97    | 92    | 94    | 93              |
| 3       | 215            | 27                     | 40                       | 33                     | 97    | 91    | 94    | 91              |
| 4       | 466            | 40                     | 68                       | 56                     | 97    | 92    | 94    | 95              |
| 5       | 391            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 97              |
| 6       | 215            | 27                     | 40                       | 33                     | 97    | 91    | 93    | 98              |
| 7       | 466            | 40                     | 68                       | 56                     | 97    | 92    | 94    | 91              |
| 8       | 391            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 92              |
| 9       | 215            | 27                     | 40                       | 33                     | 97    | 91    | 93    | 92              |
| 10      | 466            | 40                     | 68                       | 56                     | 97    | 92    | 94    | 95              |
| 11      | 391            | 33                     | 59                       | 66                     | 97    | 92    | 94    | 97              |
| 12      | 215            | 27                     | 40                       | 33                     | 97    | 91    | 93    | 91              |

**Gain of TERC in cervical adenocarcinomas**

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carcinoma in situ or invasive cervical squamous cancer in the future (Heselmeyer-Haddad et al., 2005). In the same paper, the authors were also able to show that all CIN1/CIN2 lesions with a diploid pattern for the TERC probe panel (CEP7–CEP3–TERC) eventually regressed to normal Pap smears, whereas the CIN1/CIN2 lesions with TERC amplification progressed. Lesions with a tetraploid pattern for the panel (four signals for each probe) could develop either way. The author concluded that the detection of gain and amplification of TERC in routinely collected Pap smears can assist in identifying lesions with a high progression risk. As the results of the current study indicate, TERC appears to play a similar important role in the carcinogenesis of cervical adenocarcinomas. Interestingly, even the patterns of gain found in the adenocarcinomas are very comparable to the patterns found in cervical dysplasias of squamous cell type (Heselmeyer-Haddad et al., 2003, 2005). The most common patterns observed for both of the cell types are low-level gains of TERC on a diploid background (3–5 signals of TERC). Given the similarity to the results of the studies of TERC in cervical squamous cell lesions, it seems to be a distinct possibility that analysis of TERC gain may also help to find women at risk for developing adenocarcinoma. An objective molecular marker in this patient group is of extreme importance because of the cytologically difficult identification of these cells and the limited numbers of representative cells owing to technical problems in sampling these lesions. As shown in our study, many women with cervical adenocarcinoma presented with morphologically normal Pap smears, often only 1–2 years before diagnosis. A marker like TERC could have considerable impact on the detection of cervical adenocarcinomas at an earlier stage. Fluorescence in situ hybridisation analyses of precursor lesions, that is, adenocarcinomas in situ and dysplasias will be the next step to verify the potential of TERC as a marker of early detection and progression in cervical adenocarcinomas. We are therefore planning to conduct both retrospective studies with archival Pap smears and prospective randomised studies with routinely collected Pap smears.

**Figure 2** Diagrams illustrating the scoring results from interphase FISH analyses in cases 1, 3 and 12. Scoring patterns for CEP3–CEP7–TERC are given along the x axis and the numbers of detected nuclei with a given pattern are given on the y axis.
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